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Epithelial to Mesenchymal Transition and the generation of stem-like cells in companion animal breast cancer

Alejandro Cervantes Arias

Thesis presented for degree of Doctor of Philosophy (PhD)

College of Medicine and Veterinary Medicine. The University of Edinburgh – 2016

DECLARATION

I, Alejandro Cervantes Arias, do hereby declare that the work carried out in this thesis is original, was carried out by myself or with due acknowledgement, and has not been presented for award or degree at any other university.

Alejandro Cervantes Arias Edinburgh, February, 2016

Dedication

To my uncle Jorge Arias and my grandfather José Arias. You were always my biggest example and now you are the engine of my heart.

To my parents, Francisco Cervantes and Eugenia Arias who have been there for me from day one. You are my foundation. Thank you for all your love, support, encouragement and dedication.

To my brothers Francisco Cervantes and José Cervantes. You were always in my mind during this adventure.

To my grandmother Isabel Rama. You are the strongest woman I have ever met. Thank you for everything you have done for all our family.

To Mia and Iñaki Cervantes, the youngest children in the family.

This is a tribute to all of you. Words cannot express how much I love you all.

Acknowledgements

I would like to express my deep appreciation and gratitude to my supervisor, Prof. David J. Argyle, for the patient guidance and mentorship he provided to me, all the way from the first day through to completion of this degree.

I would also like to thank Dr. Lisa Y. Pang for all the great advice she gave me. She was the scaffolding to my project.

Mrs. Rhona Muirhead, I just want you to know that you are one of the nicest persons on Earth, and none of the aims in my project would have been achieved without your help.

In a similar vein, I'd like to recognise all the people in the lab: Breno, Teresa, Thalia, Gura, Hanna, Adam, Tina, Fox, Tanveer, Craig , Julie, Mark, Tom, Joao, Karen, Mathylda, Daniela and all the staff in the Royal (Dick) School of Veterinary Studies and The Roslin Institute for the contributions that each of them made to my intellectual growth during these years. Thanks to Alejandro Cabrera for his collaboration with breast cancer images.

A special mention has to be made for my *Alma Mater*, the National Autonomous University of Mexico (UNAM) and all the staff of the Hospital for companion animals of the Faculty of Veterinary Medicine (FMVZ), who indirectly encouraged me to achieve my goals.

I would like to thank my friends in Mexico, especially the "COT" (Alvar Paz, Tarcisio Sánchez, Jorge Villegas, Emmanuel López, Christian Guerra and Emilio Gutiérrez) and Ernesto Echevarría for accepting nothing less than completion from me. To Armando Sánchez, Oscar Rico, Rafael Ojeda, Juan Nava, David Núnez, Alberto Bravo and José Antonio Ruíz; thanks for the great example you are giving as veterinarians and human beings. A big thanks to my colleagues in UVM; Carlos Castañeda and Fernando Flores for their understanding and support during my thesis corrections. Thanks to Erika Ruiz for all her support during the last stages of this thesis.

Dr. Rafael Zavala, Dr. Jesús Paredes, Dr. Carlos Santoscoy, Dra. Rosa Elena Méndez, Dr. Francisco Trigo and Dr. Carlos Federico Arias, this dream would have not become true without all your advice and encouragement to be a better person and a better veterinarian. This achievement is also yours.

To my friends in Edinburgh: Ernesto Valero, Ignacio Barajas, Andres Opazo, Adriana Mendoza, Javier Hernández, Simon Smith, Marcelo Barria, Michael Mcloughlin, Camille Longué, Shane Frankiewicz, Dulce Mendoza, Rodrigo Osorno, Stella Mazeri, Valery Madero and everyone who has made my stay in this beautiful city, the best adventure of my life. Thank you so much.

All this project was funded by the National Council of Science and Technology of Mexico (CONACyT) and the Overseas Research Students Award Scheme (ORSAS), University of Edinburgh.

Abstract

Breast cancer is the most common cancer in women and unspayed female dogs. The Epithelial to Mesenchymal Transition (EMT) is a process involved in embryogenesis, carcinogenesis, and metastasis. The Transforming Growth Factor-Beta (TGF-β) pathway and its associated transcription factors are crucial for EMT induction, during which epithelial cells lose their defining characteristics and acquire mesenchymal properties. EMT has been implicated as a driver of metastasis as it allows cells to migrate and invade different organs. Recent evidence indicates that cancer stem cells are required to establish metastatic tumours at distant sites, and that EMT may promote development of cancer cells with stem-cell characteristics, thus, the EMT pathway may be an important molecular determinant of tumour metastasis.

The main objective of this project was to characterise TGF-β-induced EMT in breast cancer models. EMT was induced by TGF-β in human, canine and feline breast cancer cell lines, and confirmed by morphological changes and molecular changes at the protein level by Western blot analysis. Changes at the mRNA level were confirmed in human and canine mammary carcinoma cell lines by qRT-PCR; migratory properties were assessed by invasion assays *in vitro* in feline and canine mammary carcinoma cells. Importantly, we observed that feline and canine mammary carcinoma cells stimulated by TGF-β acquired stem cell characteristics including sphere-forming ability, self-renewal, and resistance to apoptosis, and also enhanced migration potential. Canine cells showed resistance to chemotherapeutic drugs after TGF-β stimulation. These data suggests a link between EMT and cancer stem-cells. Moreover, global changes in microRNA expression were mapped during TGF-β-induced EMT of canine mammary carcinoma cells. This gave significant insight into the regulation of EMT in canine cancer cells and identified several potential targets, which require further investigation. During EMT cells acquire migratory properties and cancer stem-cell characteristics, suggesting that EMT and the stem-cell phenotype are closely related during cell migration and metastasis, therefore making the TGF-β pathway a potential target for the development of novel therapies against cancer and its progression.

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Abbreviations

AKT	Serine/threonine-specific protein kinase
ATP	Adenosine Triphosphate
Bax	BCL2-associated X protein
Bcl-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
BSA	Bovine serum albumine
CD	Cluster of differentiation
cDNA	Complementary DNA
Cfa-miR	Canis familiaris microRNA
CMC	Cat mammary carcinoma cell line
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CSC	Cancer stem cell
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DFS	Disease-free survival
DTT	Dithiothrietol
EDTA	Ethylene diamine tertraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to Mesenchymal Transition
ER	Oestrogen receptor

ERK	Extracellular signal-regulated kinase
FACS	Fluorescent-activated cell sorting
FBS	Foetal bovine serum
FDR	False discovery rate
FGF	Fibroblast growth factor
GF	Growth fraction
GSK3B	Glycogen synthase kinase 3 beta
HEPES	4-(2-hydroxyethil)-1-piperazineethanesulfonic acid
HER2	Human epidermal receptor 2
HMEC	Human mammary epithelial cell
HMLER	Tumorigenic breast epithelial cell
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRP	Horseradish peroxidase
IBC	Inflammatory breast carcinoma
iPSC	Induced pluripotent stem cell
JNK	c-Jun N-terminal Kinase
kDa	Kilo Dalton
Ki-67	Antigen Ki-67
KLF4	Kruppel-like factor 4
logCPM	log2 counts per million
logFC	log Fold Change
mA	Milliampere
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
Maspin	Mammary serine protease inhibitor
MCF10	Human mammary epithelial cell line
MCF7	Human breast adenocarcinoma cell line
MDT	Mass doubling time

MEF	Mouse embryonic fibroblast
MESC	Mouse embryonic stem cells
MET	Mesenchymal to epithelial transition
MIB-1	MIB-1 antibody
miR	MicroRNA
miRNA	MicroRNA
MMP-9	Matrix metallopeptidase 9
mRNA	Messenger RNA
MW	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-Kb	Nuclear factor kappa-light-chain-enhancer of B cells
NOD/SCID	Non-obese diabetic/severe combined immunodeficient
NSAIDs	Non-steroidal anti-inflammatory drugs
OCT4	Octamer-binding transcription factor 4
OHE	Ovariohysterectomy
p38	p38 mitogen-activated protein kinases
p53	Tumour suppressor protein p53
PBS	Phosphate buffered saline
PBST	PBS with Tween-20
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositide 3-kinases
PR	Progesterone receptor
qRT-PCR	Quantitative real time PCR
Rac1	Ras-related C3 botulinum toxin substrate 1
RAM	Rabbit anti-mouse antibody
Ras	Rat sarcoma viral oncogene homolog

REM	Canine mammary carcinoma cell line
Rho	Ras homolog gene family
Rhoa	Ras homolog gene family, member A
RKI	Rho-kinase inhibitor
RNA	Ribonucleic acid
RNase	Ribonuclease
ROCK	RhoA/Rho-kinase
RPL32	Ribosomal protein L32
RPM	Reads per million
RT-PCR	Reverse transcriptase PCR
SAR	Swine anti-rabbit antibody
SDS PAGE	Sodium duodecyl sulphate polyacrylamide gel
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
TEMED	Tetramethyl ethylene-di-amine
TGFBR	Transforming growth factor-beta receptor
TGF-β	Transforming growth factor-beta
TNFα	Tumour necrosis factor-alpha
TNM	Tumour, lymph node, metastasis
TRIS	Trisaminomethane
V	Volt
wd-19	TGF-β withdrawal after 19 days
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Cancer aetiology

Cancer is one of the major health problems in humans and animals. Some important characteristics of this fatal disease are the uncontrolled growth and proliferation of cells, which will frequently act against their host. Even though it is not fully understood how a regular cell is transformed into a neoplastic cell, different researchers state that the majority of cancers are originated from a single cell (reviewed by Al-Hajj and Clarke, 2004, and Reya et al., 2001). It has been thought that the initial changes in neoplastic cells are related to a deregulation of genetic mechanisms, which control cell growth, cell division and differentiation as reviewed by Dobson, 2014.

Cancer is caused by mutations which lead to resistance to apoptosis and the consequent proliferation of cells. Different genes direct and regulate several steps in the cell cycle, ensuring that there is always a balance between cell division and cell death during all stages of life, thus, damage to these genes can lead to tumour formation. In cancer, some of these genes are also known as oncogenes and tumour suppressor genes. An oncogene is a gene that can contribute to convert normal cells into cancer cells when mutated or aberrantly expressed, producing a dominant gain of function in the cells, leading to carcinogenesis (reviewed by Argyle and Khanna, 2013). Tumour suppressor genes are genes that naturally protect cells from the path of cancer and can also be called anti-oncogenes. Carcinogenesis can be initiated by mutations provoking the inhibition or loss of function of tumour suppressor genes which will facilitate progress to cancer cells, usually combined with other genetic alterations including the activation of oncogenes (reviewed by Yoshida, et al., 2000, and Weinberg, 2014).

Proto-oncogenes are cellular oncogenes that cannot transform normal cells into cancer cells on their own. They need to be altered in order to lead cells to form tumours. Many of these proto-oncogenes have developmental functions, including cell growth and proliferation (reviewed by Balmain and Brown, 1988). They can be divided into different groups such as growth factors and their receptors; protein

kinases; signal transducers; and nuclear proteins and transcription factors. The mechanisms by which these proto-oncogenes can be activated and transformed into oncogenes leading to malignancy can be achieved by chromosomal translocation, gene amplification, point mutations and viral insertions.

Some important features that can be considered as hallmarks of cancer are: the uncontrolled cell proliferation without the need of new cell formation by the organism; resistance to apoptosis; angiogenesis induction; replicative immortality; aberrant expression of proto-oncogenes stimulating neoplastic growth and overproduction of growth factors; the inactivation of tumour suppressor genes resulting in a failure to stop inadequate cell growth; cell reprogramming; and the potential to evade the immune system of the host. It is thought that at least six mutations in different genes are necessary to develop these features and consequently form the vast majority of cancers as reviewed by Hanahan and Weinberg, 2011. As a result of oncogenic mutations, regular cells can progress to neoplastic cells by the acquisition of these tumorigenic features (reviewed by Dobson, 2014).

Cancer is a multifactorial disease which can be developed by several causes, including different types of viruses, radiation, UV light, chemical products, nutritional problems and hereditary mutations among others as reviewed by Dobson et al, 2014. Examples of hereditary mutations that can cause different types of cancers include mutations of the genes BRCA1 (Nieto et al., 2003) and BRCA2 (Ochiai et al., 2001), which are important for the development of breast cancer in dogs and humans.

In humans, the tumour suppressor gene most frequently mutated is p53, a stress-activated transcription factor which is considered the guardian of the genome (reviewed by Lane, 1992). It promotes cell cycle arrest and apoptosis in DNA damaged cells, thus, stopping cell division in genetically altered cells which could possibly lead to neoplastic formation (Sluss and Jones, 2003). Mutation of this gene inhibits cell cycle arrest and apoptosis, leading to aberrant cell growth and fosters the consequent formation of different types of tumours like breast carcinoma, leukaemia,

glioma, adrenocortical carcinoma, and soft tissue sarcomas (Tabori and Malkin, 2008).

1.2 Breast cancer

Breast cancer is one of the most common types of cancer around the globe and the most common cancer among women in the UK accounting for approximately 30% of new cases every year (Cancer Research UK, 2014). According to Parkin et al (2005), there are wide variations in risk factors around the world due to the difference in exposure to environmental or lifestyle changes, thus, it becomes quite difficult to prevent the disease in different regions (Parkin et al., 2005). In 2011, there were 49,936 new cases of breast cancer in women in the UK, showing that there are approximately 155 new cases of breast cancer for every 100,000 women in the UK (Cancer Research UK, 2014).

Breast cancer is the most common cancer in unspayed female dogs constituting approximately half of all tumours in female dogs (Moe, 2001, reviewed by Sorenmo et al., 2003). In unspayed bitches, mammary tumours comprise around 50% of all types of tumours, and approximately 50% of them are malignant. Overall, breast cancer in dogs has approximately 3 times the incidence of breast cancer in humans as reviewed by Sorenmo, 2003. Both in humans and dogs mammary tumours are rich in epithelial cells and usually metastasise to the lungs or bone (Owen, 1979, Sonnenschein et al., 1991, and reviewed by Sorenmo, 2003). Canine mammary tumours share clinical and biological similarities with mammary tumours in humans, making dogs good animal models for cancer research in humans. In dogs and humans, tumours usually arise spontaneously due to genetic alterations as reviewed by Porrello et al., 2006 and Cassali et al., 2011. Moreover, mammary tumours in both species are staged according to the tumour, lymph node and metastasis (TNM) system established by the World Health Organisation (WHO) (Owen, 1980). Interestingly, skin ulcerations and local inflammatory reactions are clinical signs associated with malignant breast tumours in both dogs and humans (Cassali et al., 2011).

Kumaraguruparan et al, 2006 showed that apart from other similar gene mutations, including p53 (Rungsipipat et al., 1999), BRCA1 (Nieto et al., 2003) and BRCA2 (Ochiai et al., 2001) already described in both species by different groups, canine and human breast cancer also share pro- and anti-apoptotic molecular changes. These include increased expression of anti-apoptotic genes including B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), and 70 and 90 kilodalton heat shock proteins (Hsp 70 and 90) in both species whilst the expression of pro-apoptotic molecules like Bcl-2-associated X protein (Bax) and caspase was downregulated (Kumaraguruparan et al., 2006). These similarities give rise to the elucidation that small animals like dogs and cats could work as better animal models than rodents, which usually are immunosuppressed, for the development of novel strategies in the diagnosis and therapeutic protocols for human cancer. Moreover, they affirm that as laboratory animals do not share the same environmental factors as humans, they would not provide the same results as companion animals, which usually experience a similar lifestyle and environment as humans (Kumaraguruparan et al., 2006). Furthermore, *Gama et al, 2008* showed that canine mammary cancer is also similar to that in humans based on its molecular classification where it displays a heterogeneous panel of receptors including oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). They classified 102 canine mammary carcinomas into 4 different groups: Luminal A, luminal B, HER2 overexpressing and basal tumours. Luminal A tumours were ER+/HER2- and had the lowest proliferation rate and a better prognosis compared with the other types. They also had the highest incidence reaching 44.8%. Luminal B tumours were ER+/HER2+ with just 13.5% of incidence, and the lowest incidence was 8.3% shown by the HER2 overexpressing tumours (ER-/HER2+). Finally, the basal tumours (ER-/HER2-) had 29.2% of incidence and showed the highest proliferation rate with the shortest survival rates, similar to basal-type mammary carcinomas in humans. (Gama et al., 2008). The determination of a molecular phenotype within mammary carcinomas contributes to a better and more accurate prognosis. HER2+ carcinomas in humans can be targeted with adjuvant therapy with trastuzumab, a monoclonal antibody that inhibits the HER2/neu receptor (Slamon et al., 2011). Furthermore, as HER2 overexpression has also been identified in canine mammary carcinomas, their

therapeutic approach could be similar to that of HER2+ tumours in humans (Hsu et al., 2009, Martin de las Mulas et al., 2003).

1.3 The mammary glands of companion animals

Dogs and cats usually develop 5 and 4 pairs of mammary glands, respectively, divided in three different blocks, being M1 and M2 the cranial thoracic and caudal thoracic pairs, respectively (cats only have one pair of thoracic glands), M3 and M4 the cranial and caudal abdominal pairs, and M5 the inguinal pair of mammary glands (Evans and Christensen, 1979, Silver, 1966 and reviewed by Ettinger and Feldman, 2010). The mammary glands are vascularised by branches of the thoracic, intercostal, superficial and deep epigastric, abdominal and iliac arteries bilaterally (Evans and Christensen, 1979, Slatter, 1998). The lymphatic drainage from the thoracic pair of glands (cranial and caudal) and the abdominal cranial glands is directly connected to the axillary lymph nodes whereas the inguinal lymph nodes receive the drainage from the lymphatic vessels derived from the abdominal caudal and inguinal mammary glands. The lymphatic drainage can also be directed from the mammary glands into the abdominal and/or thoracic cavities via lymphatic vessels (Evans and Christensen, 1979, Slatter, 1998). This was confirmed by Pereira et al by lymphoscintigraphy in 2008. The aim of their study was to standardise a lymphoscintigraphy protocol by intramammary administration of 99mTc-dextran to provide a rapid lymphatic drainage examination in the mammary glands of 25 female medium-weight mongrel dogs. They observed that not only the axillary lymph nodes were involved in the lymphatic drainage from the thoracic and cranial abdominal pairs of glands, but also the superficial cervical, the cranial sternal and the accessory axillary lymph nodes were radioactive after the administration of the radionuclide. Furthermore, they showed that both, the caudal abdominal and the inguinal pairs of glands were drained by the superficial inguinal and simultaneously by the medial iliac lymph nodes (Pereira et al., 2008). Interestingly, important variations were found among their groups of study. They observed radioactivity in the sternal lymph node after administration of the radionuclide in the caudal thoracic mammary glands

in 60% of the bitches in their experimental group. This pair of mammary glands was considered to be exclusively drained towards the axillary lymph node by the previously mentioned authors. They also identified important variations regarding the lymphatic drainage from the caudal abdominal mammary glands. They observed radioactivity in cervical, cranial mediastinal, superficial inguinal and medial iliac lymph nodes after injection in the caudal abdominal glands. These findings brought to their attention the need to perform individualised lymphoscintigraphy protocols for each patient when lymphatic drainage is of clinical relevance for therapeutic planning and confirmation of regional metastasis (Pereira et al., 2008).

1.4 Canine mammary gland tumours

In developing countries, where the practice of ovariohysterectomy (OHE) is not common in dogs, mammary tumours have a remarkably high incidence, making breast cancer the most common type of cancer in bitches (reviewed by Sorenmo, 2003). Vascellari et al., 2009 showed that 56% of dogs with diagnosed cancer have breast cancer, and interestingly, other studies have described that similar to humans, the male population in dogs, compared with females, has a very low incidence in which the potential risk of developing breast cancer is lower than 1% (reviewed by Saba et al., 2007, Todorova, 2006, Vascellari et al., 2009). According to Perez-Alenza et al., 2000, increased age; irregular oestrous cycles; cystic endometrial hyperplasia; and late or a lack of ovariohysterectomy history would be major risk factors associated with breast cancer development in small animals (reviewed by Perez-Alenza et al., 2000).

It has been well established that, similar to mammary neoplasia formation in humans, sexual hormones oestrogen and progesterone contribute to the development of breast cancer in dogs (Munson and Moresco, 2007), thus, according to Schneider's findings more than 40 years ago, canine breast cancer can be prevented by performing OHE before the first oestrus cycle of the bitch, as these individuals would only have $\leq 0.5\%$ probability to develop a mammary tumour (Schneider, 1970, Schneider et al., 1969).

1.4.1 Clinical aspects and diagnosis of canine mammary tumours

After a physical examination in which a growing mass is located in any of the 5 pairs of mammary glands, a histological diagnosis is needed to establish the type of tumour and if it has a benign or malignant character. Mammary tumours can be solitary, but they can also be presented as multiple masses invading one or more mammary glands (Image 1.1). Some important clinical parameters to associate canine mammary tumours with lower chance of survival and worse prognosis include large tumour size, ulceration of skin and adherence to surrounding tissues, lymph node invasion, irregular shape and increased age at the time of diagnosis as reviewed by Perez-Alenza et al., 2000. Three view thoracic radiography and sampling of lymph nodes should be performed to rule out metastasis after a mammary tumour has been diagnosed (Image 1.2), as approximately 50% of canine mammary tumours metastasise to regional lymph nodes and lungs as reviewed by Nelson, 2000 and Ogilvie, 2006.

Image 1.1. Unspayed female dog with a mammary carcinoma presented as multiple masses. Image courtesy of Alejandro Cabrera Bedolla, Clinica Veterinaria de Especialidades de Morelos, Mexico, 2013.

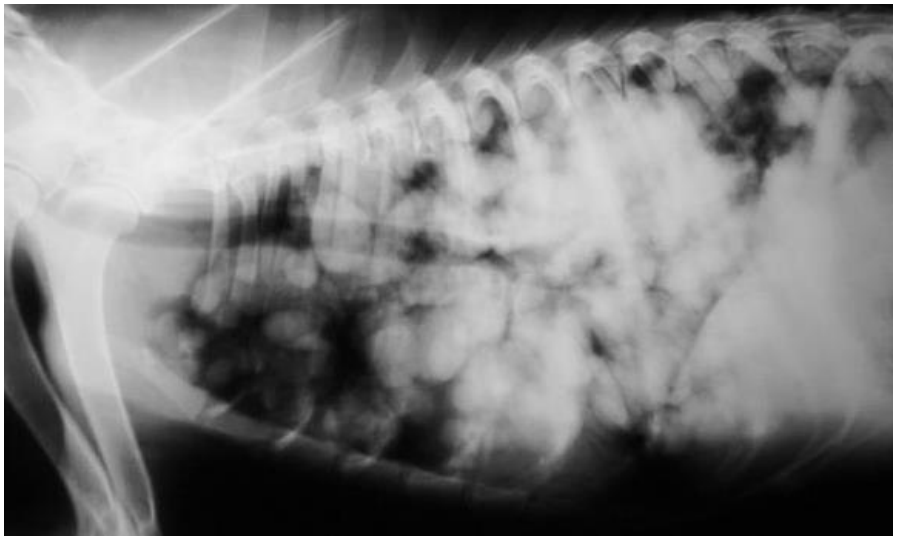

Image 1.2. Right lateral view of a thoracic radiography of an unspayed female dog with mammary carcinoma in which multiple pulmonary nodules are visible, creating a nodular interstitial pattern. These radiographic signs are compatible with advanced lung metastasis (no cytologic or histopathologic diagnosis was performed for this case). Image courtesy of Alejandro Cabrera Bedolla, Clinica Veterinaria de Especialidades de Morelos, Mexico, 2013.

Furthermore according to Rutterman et al., 2001, mammary tumours in dogs and cats can be staged through a TNM (Tumour, lymph node and metastasis) modified classification system similar to that of the World Health Organisation (WHO) system for mammary tumours in humans (reviewed by Rutteman et al., 2001). In this modified TNM system, tumours are classified in 5 stages according to the size of the tumour and lymph node involvement and distant metastasis. The letter T stands for tumour and it has 3 stages, T1 when the tumour is <3 cm in diameter, T2 for tumours measuring 3-5 cm and T3 for tumours larger than 5 cm. If there is or there is not lymph node involvement the tumour would be classified as N1 or N0, respectively, and similarly for distant metastasis, the tumour would be classified as M0 if there are no signs of metastasis or M1 if distant metastasis is diagnosed. The 5 different stages in this modified TNM system can be determined by a combination of any of these different factors previously described as shown in Table 1.1.

Stage	Tumour size	Lymph node involvement	Distant metastasis
Stage I	T1 <3 cm	N0 (negative)	M0 (negative)
Stage II	T2 3-5 cm	N0 (negative)	M0 (negative)
Stage III	T3 >5 cm	N0 (negative)	M0 (negative)
Stage IV	T1-T3	**N1 lymph node involved**	M0 (negative)
Stage V	T1-T3	N0-N1	**M1 metastasis**

Table 1.1. Modified TNM classification for mammary tumours in dogs according to the World Health Organisation to classify different stages of breast cancer in humans.

Different classification systems based on the recommendations of the WHO have been described in the past, where canine mammary tumours can be separated by histopathological origin and prognosis (Misdorp, 1999, Hampe and Misdorp, 1974), and recently Goldschmidt et al., 2011, proposed a more accurate histological classification using the previous papers as a base (Goldschmidt et al., 2011). Tumour growth in the canine mammary glands can therefore be divided into different categories depending on their histopathological origin and malignancy. Benign mammary neoplasms are divided into several types, including adenomas (ductal, intraductal papillary), fibroadenoma, myoepithelioma and adenomyoepithelioma (complex adenoma) (Goldschmidt et al., 2011). Interestingly, a study carried out by Soremno et al. in 2009 shows how, similarly to human mammary neoplasia, benign tumours in dogs can progress to a malignant phenotype (Sorenmo et al., 2009).

The tumour classification proposed by *Goldschmidt et al., 2011*, is shown in Table 1.2 (Goldschmidt et al., 2011), emphasising in malignant epithelial tumours.

1. Malignant Epithelial neoplasms	
Carcinoma *in situ*	Well demarcated nodules without involvement of surrounding tissue.
Simple carcinoma	a) Tubular (cells arranged in tubules) b) Tubulopapillary (tubules are arranged in a papillary fashion) c) Cystic-papillary (papillae extend into cystic tubular lamina) d) Cribiform (neoplastic cells form a sieve-like arrangement)
Micropapillary invasive carcinoma	One or more nodules with intraductal neoplastic cells forming small intraluminal aggregates and papillae.
Solid carcinoma	Solid masses of cells without lumina.
Comedocarcinoma	Necrotic areas in the centre of the neoplastic cell aggregates.
Anaplastic carcinoma	Most malignant of mammary carcinomas. Neoplastic cells usually invade interlobular connective tissue and lymphatic vessels.
Carcinoma arising in a complex adenoma/mixed tumor	Either a complex adenoma or a mixed tumour, with areas of more pleomorphic epithelial cells with increased numbers of mitoses.
Complex carcinoma	A malignant epithelial component and a benign myoepithelial component.
Carcinoma and malignant myoepithelioma	Two cell populations in which both, the epithelial and myoepithelial components are malignant.
Mixed carcinoma	Uncommon neoplasia with a malignant epithelial component and a benign mesenchymal component.
Ductal carcinoma	Neoplastic cells arranged in cords and tubules lined by a double layer of epithelial cells.
Intraductal papillary carcinoma	Proliferation of a multilayered population of epithelial cells. Papillae are supported by fibrous connective tissue and myoepithelial cells.
2. Malignant epithelial neoplasms – special types	Squamous cell carcinoma Adenosquamous carcinoma Mucinous carcinoma Lipid-rich (secretory) carcinoma Spindle cell carcinomas Inflammatory carcinoma

Continued overleaf.

3. Malignant mesenchymal neoplasms - Sarcomas	Osteosarcoma Chondrosarcoma Fibrosarcoma Hemangiosarcoma Other sarcomas
4. Carcinosarcoma – Malignant mixed mammary tumour	Composed partly of epithelial cells and connective tissue elements, both malignant. Epithelial cells metastasise via lymphatic vessels to regional lymph nodes and lungs. Mesenchymal cells migrate through blood vessels to the lungs.

Table 1.2. Classification of canine mammary tumours proposed by Goldshmidt et al., 2011. This table emphasises the malignant epithelial neoplasms.

1.4.2 Prognosis

According to *Sorenmo et al., 2011 and 2013*, the following 3 prognostic factors are the most consistently associated with canine mammary cancer prognosis (Sorenmo et al., 2013, Sorenmo et al., 2011):

1) Tumour volume; *MacEwen et al., 1985,* showed that the most significant prognostic factor in a study with 144 dogs diagnosed with malignant mammary tumours, was the volume of the masses, where tumours smaller than 41 cc (approximately 3.4 cm) had statistically significant better prognosis in terms of survival time than those bigger than 41 cc (MacEwen et al., 1985).

2) Lymph node status; different studies have shown that lymph node invasion is highly prognostic and important for consideration of therapeutic protocols after surgery (reviewed by Kurzman and Gilbertson, 1986, Szczubial and Lopuszynski, 2011).

3) TNM staging; tumour size and lymph node status are parameters included in the TNM staging system, both of which provide important information regarding mammary cancer prognosis. In two separate studies, *Yamagami et al., 1996* and *Philibert et al., 2003,* observed that when combining the TNM staging with the WHO histological grading system in 175 and 99 bitches, respectively, the results of the evaluations were determinant for prognosis. This data could be useful for the prognostic determination in canine mammary tumours (Yamagami et al., 1996, reviewed by Philibert et al., 2003).

1.4.2.1 Molecular markers of mammary carcinoma

Mammary carcinoma in women and bitches is highly curable if diagnosed at early stages; thus, it is of great importance to identify molecular biomarkers as potential prognostic factors.

A number of potential biomarkers for mammary carcinoma have been widely studied. Common molecules usually utilised to identify cancer patients with early stages of breast cancer include markers of proliferation, hormone receptors and the human epidermal factor receptor (EGFR) (Esteva and Hortobagyi, 2004). Cell proliferation rate is one of the most important prognostic factors in breast cancer and Ki-67 is a nuclear antigen commonly used as a proliferation marker. It is expressed during M phase of the cell cycle (proliferation phase), and overexpression of Ki-67 in patients with breast cancer has been associated with tumour recurrence (Veronese et al., 1993). In breast cancer, Ki-67 expression has also been correlated with expression of other proliferation markers, including the proliferating cell nuclear antigen (PCNA) (Keshgegian and Cnaan, 1995).

Hormone receptors such as ER and progesterone receptor (PR) are also well-established prognostic factors for breast cancer in humans. Overexpression of ER has been associated with longer disease-free survival (DFS) rate, where patients with ER+ breast cancer had a longer DFS compared to ER- patients (Thorpe et al., 1986). As PR has a close relation with ER, its overexpression would indicate that the ER pathway is intact and the patient's prognosis would be as if the tumour was ER+ (Esteva and Hortobagyi, 2004).

Human Epidermal Growth Factor Receptor 2 (HER-2), a member of the EGFR family, is important during breast cancer pathogenesis, and is overexpressed in breast cancer patients (Slamon et al., 1987). HER-2 overexpression is associated with shorter disease-free survival and death in node-positive patients (Borg et al., 1990).

Matrix Metallopeptidase-9 (MMP-9), a zinc-dependent endopeptidase of a large family of matrix metallopeptidases, has also been found to be a valuable

prognostic factor in breast cancer in humans. MMP-9 has a broad capacity for tissue remodelling by degrading the basement membrane and inducing angiogenesis, which in part can trigger tumour invasion and metastasis (Nowac et al., 2008). It has been demonstrated that MMP-9 overexpression in stromal cells is a predictive factor for poor survival in small breast tumours, but interestingly, the expression of this enzyme is associated with survival in breast carcinoma cells (Pellikainen et al., 2004). In the need for novel therapies against cancer, MMP-9 has been targeted previously with disappointing results due to toxicity produced by MMP inhibitors as reviewed by Fingleton, 2008. Perhaps because some MMPs act as protective enzymes, the indiscriminate inhibition of MMPs can produce musculoskeletal toxicity (Sparano et al., 2004 and reviewed by Decock et al., 2011). New approaches for MMP-9 inhibition include small molecules that inhibit gelatinases (Kruger et al., 2005) and subsets of MMP-9 substrate (Bjorklund et al., 2004), as well as monoclonal antibodies targeting the catalytic domain of MMP-9 (Sela-Passwell et al., 2012).

In a similar approach, the identification of molecular markers in canine breast cancer can prove useful for the determination of prognosis, but also to establish therapeutic targets. Among these markers, Ki-67 and PCNA have been studied and confirmed to have prognostic value. Pena et al., 1998 observed that their expression was directly proportional to malignancy and metastasis in canine mammary tumours. Also in benign tumours and dysplasias, these proteins were poorly expressed compared to malignant tumours (Pena et al., 1998). Santos et al., 2013 later confirmed these observations and moreover, it was found that MMP-9 was also associated with malignancy in canine mammary tumours, and consistent with the human disease (Pellikainen et al., 2004), suggesting that these molecules could be potential targets after surgical treatment (Santos et al., 2013).

p53 is the most frequently mutated tumour suppressor gene in cancer in humans as reviewed by Vogelstein et al., 2010. Veldhoen et al., 1999 and Lee et al., 2004 showed that p53 mutation and overexpression of its protein were also associated with malignancy and poor prognosis in canine mammary tumours, as in humans (Veldhoen et al., 1999, Lee et al., 2004). These findings could prompt

further research with new experimental models to elucidate protective mechanisms to prevent this tumour suppressor gene from being mutated in companion animals.

1.4.3 Treatment

For canine mammary cancer, treatment can be divided into surgical and systemic. The treatment of choice is surgical removal of the mass or multiple masses, except for those patients diagnosed with distant metastasis or with inflammatory carcinoma as reviewed by Sorenmo et al., 2013. Adjuvant systemic treatment, including chemotherapy, radiotherapy and hormonal therapy can be recommended in dogs with high-risk tumours with aggressive histology and positive lymph nodes, where surgery alone is not enough to treat them (Sorenmo et al., 2013). Even though surgery will always be the first option, there is a lack of prospective clinical trials in dogs that could elucidate the benefits of different "doses" or extent of surgical excision. The owner must be aware of the goals of the surgical procedure and discuss the aims of the surgery with the veterinary surgeon as there are several surgical techniques, including simple lumpectomy or nodulectomy, mastectomy, regional mastectomy, chain mastectomy and bilateral mastectomies (reviewed by Sorenmo et al., 2013). Lana et al., 2007 recommend specific surgical approaches depending on the extent of the tumour. For tumours smaller than 0.5 cm, they suggest a simple lumpectomy, but if the tumour is $\leq$ 1 cm with ulcerated skin or adhesions, a mastectomy is recommended. For larger tumours either a simple mastectomy with wide excision, or a regional or a chain mastectomy would be recommended depending on the extension of the tumour and if there are multiple masses. Wide excisions should have at least 2 cm lateral margins and in some cases with deeper tumours, the abdominal muscular fascia and parts of the abdominal wall should be included in the excision as reviewed by Lana et al., 2007 and Sorenmo et al., 2013. However, different retrospective studies have shown that there is no difference in overall survival time and disease-free interval after simple mastectomy or chain mastectomy (MacEwen et al., 1985, Chang et al., 2005).

Systemic treatment is usually recommended for high-risk tumours although there is no clinical evidence of its efficacy due to the lack of adequate prospective

randomised trials with different therapeutic protocols, reviewed by Sorenmo et al., 2013. Systemic treatment protocols include hormonal therapy, and most commonly chemotherapy, usually combined with nonsteroidal anti-inflammatory drugs (NSAIDs) (Marconato et al., 2009, De M. Souza et al., 2009). Karayannopulou et al., 2001 carried out a prospective non-randomised trial on 16 bitches with high risk mammary tumours, where they separated 2 groups of 8 patients. Both groups had had surgical treatment, but only one of the groups received adjuvant chemotherapy with a combination of 5-fluorouracil and cyclophosphamide, and they observed that the chemotherapy treated group had longer disease-free interval and survival time; and even though most of the patients had a decrease in leucocyte counts, they remained within normal limits except for one bitch that presented temporary leukopenia (Karayannopoulou et al., 2001). Nevertheless, as this study utilised a small group of patients, it may have a low impact as a clinical trial, but it still suggests that chemotherapy as adjuvant treatment might be useful for high-risk tumours. More prospective studies are needed to determine proper and specific chemotherapeutic protocols for different types of mammary tumours in dogs.

Two different retrospective studies confirmed that the use of NSAIDs alone or in combination with chemotherapy to treat canine mammary inflammatory carcinoma was beneficial in terms of survival time (Marconato et al., 2009, De M. Souza et al., 2009). These effects were also confirmed *in vitro* by observing cytotoxic effects in a canine mammary carcinoma cell line after treatment with the NSAIDs piroxicam and deracoxib, suggesting that cyclooxygenase-2 (COX-2) inhibition might be a useful approach for the development of novel therapeutic protocols against canine mammary cancer (Ustun Alkan et al., 2012). Moreover, Pang et al., 2014 showed that mavacoxib, a long-acting COX-2 inhibitor has a direct anti-proliferative effect in canine cancer cells, including osteosarcoma, glioma, lymphoma, mast cell tumour and hemangiosarcoma cells *in vitro*. They also proved that this NSAID has an inhibitory effect on cell migration and a pro-apoptotic effect on osteosarcoma cell lines; and more importantly, osteosarcoma-derived cancer stem cells were sensitive to its cytotoxic effects, making this drug a potential therapeutic candidate for canine cancer treatment (Pang et al., 2014).

Tamoxifen citrate has been used in humans as a selective inhibitor of oestrogen receptors in ER+ breast cancer in women (Jordan, 2006) but there is no evidence of its clinical relevance in dogs. It has been associated with adverse effects regarding hormonal disparity between oestrogen and progesterone in intact bitches that could potentially lead to the development of pyometra (Tavares et al., 2010). Thus, it is not a standard care in un-spayed female dogs.

There is still much to be elucidated regarding the use of systemic therapeutic protocols alone or in combination with other treatments, and thus, there is no consensus that they are clinically relevant, even in cases with lymph node metastasis or distant metastasis. Currently the best way to combat mammary tumours in dogs is preventive by performing OHE at early stages of life (Schneider, 1970, Schneider et al., 1969).

1.5 Introduction to EMT

Epithelial to mesenchymal transition (EMT) is a process through which epithelial cells lose their characteristics and acquire mesenchymal properties. This process is rapid and reversible (reviewed by Klymkowsky and Savagner, 2009) and can facilitate cell migration. The EMT program is essential throughout different embryonic stages, including organogenesis and gastrulation. Cells acquiring mesenchymal characteristics are able to move towards different places in the embryo in order to generate organs and their anatomical structures as reviewed by Shook and Keller, 2003, and Thiery, 2003. This process, by increasing cell motility and invasiveness, plays roles in wound healing, and metastasis. Based upon the fundamental roles that EMT has, this process has been classified as follows:

Type I	Embryogenesis and organ development
Type II	Fibrosis - wound healing and tissue regeneration
Type III	Metastasis – enabling neoplastic cells to invade different tissues and organs during cancer progression

Epithelial to mesenchymal transition mediates reorganisation of the cytoskeleton by decreasing cell-cell contact and changing cell polarity as reviewed by *Acloque et al., 2009*. Epithelial cells adhere to each other by lateral cell-cell junctions. These cells are polarised such that the bottom is defined as basal, and the top as apical. The matrix binding areas are located at the basal aspect of the epithelium. Due to these properties, epithelial cells generally form groups with tight junctions. In contrast, as shown in Figure 1.1, mesenchymal cells have a more elongated shape and their polarity is reversed, therefore they do not form large groups, and easily migrate (reviewed by Acloque et al., 2009). These changes are induced by transcriptional repression of proteins, such as cadherins, occludin, claudin and desmoplakin. This repression can be accomplished by a range of transcription factors including ZEB1, Snail, Slug and Twist. Furthermore, epithelial cells undergo cytoskeletal changes, acquiring increased motility and invasiveness (reviewed by Savagner et al., 1994, and Thiery and Chopin, 1999).

If a cell is to achieve colonisation of a distant site, then EMT has to be reversed. This reversion is called Mesenchymal to Epithelial Transition (MET), and without it, cells would not be able to form any tissue or organ, including any kind of metastatic colonisation as reviewed by Lee et al., 2006. After MET, cells gain back their lost polarity and adhere to each other by tight junctions. This is due to an upregulation of proteins that help create adherens junctions such as E-cadherin and β-catenin, and the downregulation of those which can enable cells to break their adhesions and change their shape and polarity, such as fibronectin and vimentin (Zeisberg et al., 2005). All three sub-types of EMT (Types I-III) require the reverse Mesenchymal-Epithelial Transition (MET) program to enable cells to attach to their new microenvironments and establish a niche as reviewed by *Kalluri and Weinberg, 2009.*

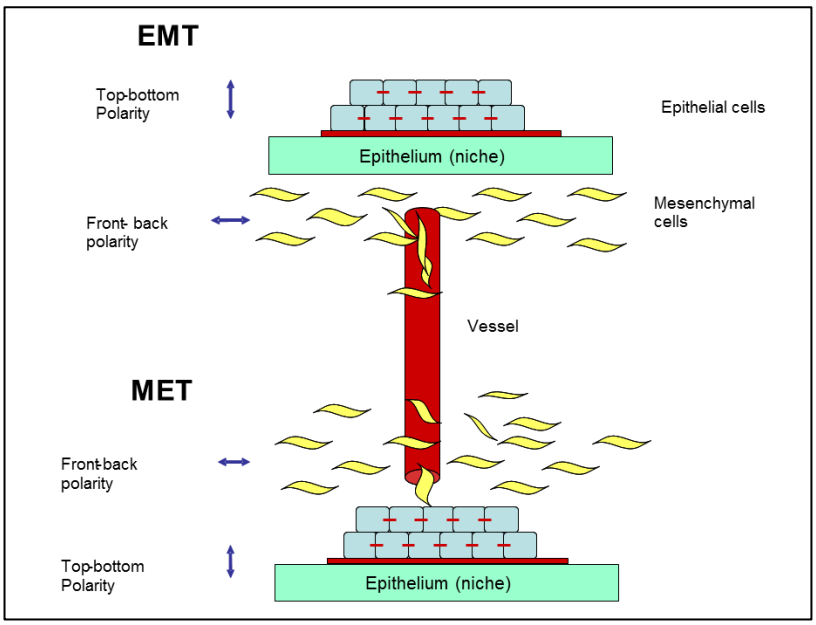

Figure 1.1. Polarity changes in cells undergoing EMT and the reverse program MET. Epithelial cells acquire front-back polarity and lose cell-cell contact, becoming mesenchymal and migrating through surrounding tissues and blood and lymphatic vessels. Once they reached the new microenvironment (niche), they regain their epithelial characteristics by MET.

This process is relevant in cancer progression, as in some cancers with epithelial origin like skin, stomach or breast cancer, cells would have to go through an EMT process in order to migrate and invade a different organ as reviewed by *Kalluri and Weinberg, 2009*. This process is part of metastatic progression where a primary tumour evolves and produces a secondary or metastatic tumour, which in most cases can be lethal. During EMT, cells acquire migratory properties and cancer stem-cell characteristics (reviewed by Polyak and Weinberg, 2009, Morel et al., 2008, Mani et al., 2008), suggesting that EMT and the stem-cell phenotype are closely related during cell migration and invasion. The EMT process has been implicated as a driver of metastasis (reviewed by Kalluri and Weinberg, 2009).

1.6 Metastatic progression

The molecular mechanisms and pathogenesis underlying metastasis are not fully understood yet. Undoubtedly, a better understanding of these mechanisms would help create novel therapeutic protocols against cancer and its progression (Smit et al., 2009).

Tumour metastatic progression has been considered the final stage of cancer, which causes approximately 90% of cancer deaths (reviewed by Im et al., 2012, and Wu and Zhou, 2008). It is a complex, multistep process in which malignant tumour cells migrate through surrounding tissues and invade distant sites where they can spread and attach to different organs creating a secondary tumour as reviewed by *Kusewitt and Rush, 2007*. Four distinct steps have been named to describe this process: invasion, intravasation, extravasation, and metastatic colonisation (reviewed by Chambers et al., 2002, Pantel and Brakenhoff, 2004).

It has been suggested that the presence of the primary tumour modulates the microenvironment, also called niche, of the site where metastatic cells will attach, prior to the arrival of most of them. Once cells arrive at their new niche, they must survive and proliferate to start modulating it to their convenience, a process that would be necessary for a metastatic tumour to grow and progress within its surrounding stroma (reviewed by Kaplan et al., 2006). The tumour itself can modify this stroma, and interestingly, Transforming growth factor-Beta (TGF-β) may play a crucial role during the metastatic niche modulation by stimulating angiogenesis and recruiting different types of host cells, including myofibroblasts, in order to prepare the "scaffolding" for the metastatic tumour (De Wever and Mareel, 2003). Interestingly, Twist and Snail (transcription factors with important roles in the TGF-β pathway) have been associated with tumour progression, by suppressing/increasing resistance to anoikis, thus, contributing to metastasis (Smit et al., 2009). Therefore, a tumour can be considered as a complex system which supports the malignant characteristics that lead to metastasis. This complex system is composed by cancer cells and cancer stem cells in the first place, followed by endothelial cells, pericytes,

immune cells and tumour-associated fibroblasts as reviewed by *Argyle and Khanna, 2013*.

Stephen Paget first suggested in 1889 that tumour cells grew preferentially in specific organs due to their microenvironment interacting with colonising cells. He called this "the seed and soil hypothesis", where the tumour cells would be the seed and the microenvironment of these specific organs would be the soil (Paget, 1889). This hypothesis has been supported by different research groups, for instance, in 1960*; Kinsey* demonstrated that lung melanoma cells in syngeneic mice, migrated to normal and ectopically placed lung tissues, but not to other places around the organism (Kinsey, 1960). Furthermore, in 1988, *Schackert and Fidler* showed that mouse melanoma and fibrosarcoma cells metastasised to specific organ regions within the central nervous system (Schackert and Fidler, 1988). However, other studies reported that metastasis was not due to the seed and soil hypothesis, but to a specific anatomical site of the primary tumour and the vascular and lymphatic drainage leading to the first organ encountered (Ewing, J., 1928). Regarding these hypotheses, *Langley and Fidler, 2011* concluded that neither of them is necessarily exclusive of each other (Langley and Fidler, 2011).

1.7 Mammary carcinoma metastasis

Metastatic potential is very high among mammary carcinomas from which metastatic cells migrate and invade different organs, where they will colonise, and be more aggressive than the primary tumour. In humans, the primary site for breast cancer metastasis is bone, followed by lungs (Chen et al., 2010, Coleman, 2006). In canine and feline breast cancer, the primary metastatic site is the lungs (reviewed by Nelson, 2000, Ogilvie, 2006). Canine mammary carcinoma is the most common cancer among female dogs and is often fatal due to the development of distant metastases, reviewed by *Von Euler, 2014*. Moreover, in bitches, 50% of mammary tumours are malignant, and half of them usually progress and metastasise to distant organs (reviewed by Argyle et al., 2008).

Lymph node metastasis is considered the first step in the metastatic cascade of mammary carcinoma (Olmeda et al., 2007b), and is one of the most well founded factors for predicting a poor prognosis (Fisher et al., 1978) due to the fact that distant metastases are not common findings at presentation (Wingo et al., 1995). Most mammary carcinomas in dogs metastasise through lymphatic vessels. The most cranial pairs of glands (M1 and M2) are typically drained through lymphatic vessels to the axillary lymph nodes. The third pair (M3) can be drained by the axillary and inguinal lymph nodes, whilst the two most caudal pairs (M4 and M5) typically drain towards the inguinal lymph nodes. Regional lymph nodes metastasis usually leads to a worse prognosis as reviewed by *Von Euler, 2014*. As described above, the lungs are the most affected organs after lymphatic invasion in dogs, thus, it is always recommended to perform three view thoracic radiography to rule out lung metastasis prior to any therapeutic protocol, although in 2003, *Djupsjobacka* and *Eksell* observed that in dogs younger than 8 years of age with a metastatic pulmonary tumour measuring 1 cm or less, the possibility to detect thoracic metastases was only 3% (Djupsjobacka and Eksell, 2003). Distant metastasis can also involve other organs, including liver, kidneys, spleen, bones, central nervous system and pleura as reviewed by *Von Euler, 2014.*

1.8 EMT in cancer and during metastasis

Recent data show that EMT is commonly observed in primary tumours at their cancer invasion front (reviewed by De Weber, 2008). It is also associated with resistance to anticancer agents such as EGFR inhibitors (Voulgari and Pintzas, 2009). During EMT III cancer cells follow similar pathways as normal cells during embryonic development and wound healing. The existence of a reversible program (MET) which would enable cells to form a new epithelial sheet has been proposed by *Thiery, 2002* (Thiery, 2002) and *Hugo et al., 2007* (Hugo et al., 2007). Some cancer cells may also pass through just a partial EMT, expressing both, mesenchymal and epithelial markers during the process as reviewed by *Yang and Weinberg, 2008*.

There are three important pathways in EMT: wingless-related integration site/β-catenin (Wnt/β-catenin); fibroblast growth factor (FGF); and transforming growth factor-beta 1/Bone morphogenetic protein (TGFβ1/BMP). These pathways are vital for embryogenesis, and also play important roles in tumour formation and progression. The transforming growth factor β1 (TGFβ1) pathway is crucial in EMT induction due to its multiple downstream effectors capable of repressing E-cadherin and subsequently enabling upregulation of mesenchymal promoters, such as fibronectin and vimentin as reviewed by *Thiery and Sleeman, 2006* and *Oft et al., 2002*.

1.9 Molecular features of EMT during tumour progression

There are four important stages in cancer development and cell migration which are 1) Invasion, 2) Intravasation, 3) Extravasation, and 4) Metastatic colonisation (Weinberg, 2008). In order for an epithelial tumour to complete each step during this process, molecular changes are required to enable EMT.

One of the principal characteristics of EMT is the loss of E-cadherin. This protein participates in cell-cell adhesion and interacts with other molecules to form epithelial junctions. It is a calcium-dependent cell adhesion molecule that connects epithelial cells by calcium-dependent homotypic interactions as reviewed by *Schmalhofer et al., 2009*, playing an important role in the maintenance of epithelial layers (Matos et al., 2006). The expression of E-cadherin is inversely proportional to tumour grade and stage, and patient prognosis as reviewed by *Schmalhofer et al., 2009*. β-catenin is also essential for keeping cells attached to each other, it acts by binding E-cadherin and α-catenin to the actin cytoskeleton as depicted in Figure 1.2 (reviewed by Schmalhofer et al., 2009, Acloque et al., 2008 and Brabletz et al., 2005).

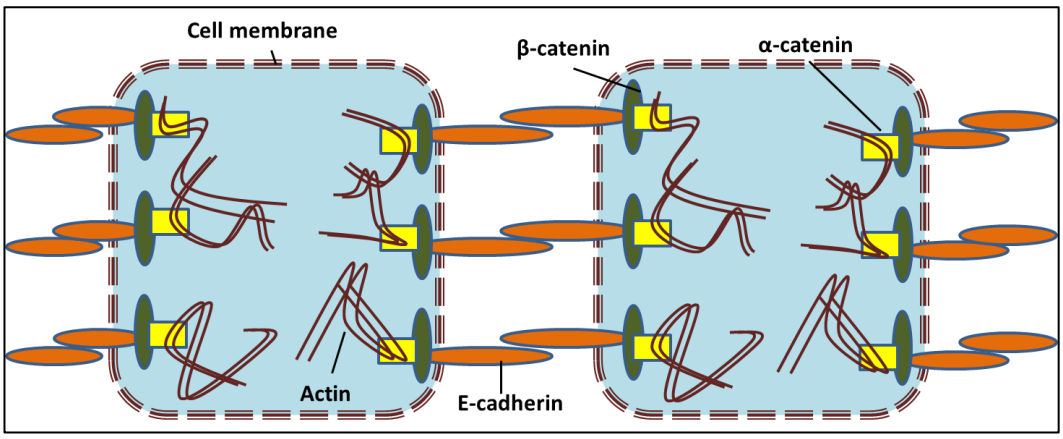

Figure 1.2. The extracellular domains of E-cadherin homodimers are connected by calcium-dependent homotypic interactions with those of dimers on adjacent cells. The intracellular domain of E-cadherin attaches to β-catenin and α-catenin to interact with the actin cytoskeleton and form the adherens junctions.

Once epithelial cells detach from other cells and their epithelial sheet, they can acquire motility by changing their shape and polarity in order to migrate through different tissues, and lymphatic and blood vessels as reviewed by *Thiery, 2003*. Vimentin is an intermediate filament protein with structural features, which enables it to play a main role in switching the cell's shape and making its cytoskeleton stronger so it can be more flexible and prevent damage to the cell membrane (reviewed by Goldman et al., 1996). It binds with microtubules and actin microfilaments to make up the cytoskeleton, without this protein migrating cells would be very fragile. Not only is a robust cytoskeleton necessary for migrating cells; it is also necessary to guide these cells during migration as reviewed by *Goldman et al., 1996*. Fibronectin is a high molecular weight extracellular matrix glycoprotein that plays important roles in cell adhesion, growth, migration and differentiation (Williams et al., 2008), by guiding cells and binding to collagen and fibrin. Genetic alterations in fibronectin have been associated with pathologies, including fibrosis and metastasis (Darribere and Schwarzbauer, 2000, Hao et al., 2004, Williams et al., 2008).

These molecular alterations are due to different transcription factors, which act as EMT inducers by stimulating and/or suppressing functions of different proteins

involved in this complex process. Snail, Slug (Snail 2), Twist and ZEB1 are important transcription factors involved in EMT (Mathias et al., 2009). They bind to the proximal E-boxes of the promoters of E-cadherin in order to contribute to its transcriptional repression, considered to be the hallmark of EMT (Bolos et al., 2003). The Snail family is made up of Snail and Slug (Snail 2) and they are involved in different stages of development such as gastrulation, mesoderm formation, cell differentiation, cell motility and apoptosis (Boulay et al., 1987, Seki et al., 2003 and reviewed by Hemavathy et al., 2000 and Arias, 2001). They are implicated in EMT by downregulation of expression of E-cadherin (Sugimachi et al., 2003, Hajra et al., 2002, Bolos et al., 2003). *Olmeda et al., 2007b* showed that when Snail and Slug were inhibited in mammary tumour cell lines, tumour growth was impaired and their metastatic potential was lowered in mice (Olmeda et al., 2007b). Twist is part of the basic helix-loop-helix protein family and one of its most important features is the ability to inhibit apoptosis (Maestro et al., 1999). It is also known to trigger EMT by downregulating E-cadherin expression (Yang et al., 2004). Two members of the zinc finger E-box binding proteins family are ZEB1 and ZEB2, both of which can regulate E-cadherin and thus, are capable of stimulating EMT as depicted in Figure 1.3 (reviewed by Peinado et al., 2007, Eger et al., 2005, Comijn et al., 2001). *Burk et al., 2008* showed that ZEB1 was overexpressed in colorectal, pancreatic and breast cancer cell lines undergoing EMT induced by TGFβ1 and TNFα (Burk et al., 2008). Moreover, when ZEB1 was knocked down by siRNA, EMT was partially prevented. Each of these different epithelial/mesenchymal biomarkers and transcription factors has different characteristics and functions as shown in Table 1.3.

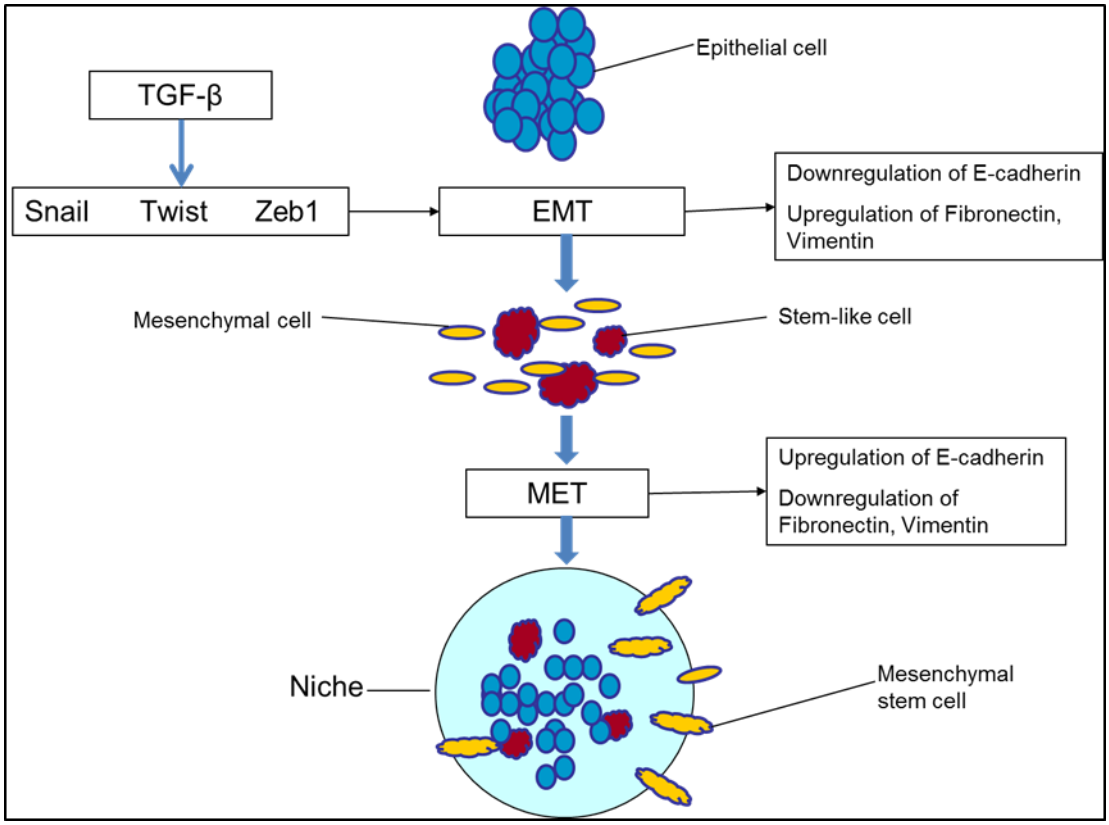

Figure 1.3. EMT induces cells to acquire stem cell-like characteristics. After undergoing EMT, epithelial cancer cells acquire not only mesenchymal characteristics like spindle shape and front-back polarity, but also stem cell-like features such as tumour seeding abilities and resistance to apoptosis. However, once they reach their new microenvironment, they undergo Mesenchymal-Epithelial transition (MET) in order to be capable of colonising it.

Protein / transcription factor	Origin	Characteristics	Expression during EMT	References
E-cadherin	Epithelial	Cell-cell adhesion. Tumour suppressor gene.	Downregulation	(Schmalhofer et al., 2009, Yang et al., 2004, Hajra et al., 2002, Peinado et al., 2007)
β-catenin	Epithelial	Adherens junctions. Transmits the contact inhibition signal once the epithelial sheet is complete.	Downregulation	(Schmalhofer et al., 2009, Acloque et al., 2008)
Fibronectin	Mesenchymal	Binds extracellular matrix components such as collagen and fibrin. Growth, migration, differentiation, wound healing. Cancer and fibrosis.	Upregulation	(Thiery and Sleeman, 2006, Yang et al., 2004, Zeisberg and Neilson, 2009)
Vimentin	Mesenchymal	Make up the cytoskeleton. Supports the position of organelles in cytosol. Maintains cell shape and offers flexibility to the cell. Strengthens the cytoskeleton.	Upregulation	(Thiery and Sleeman, 2006, Yang et al., 2004, Burk et al., 2008, Zeisberg and Neilson, 2009)
Snail		Critical for mesodermal development. E-cadherin repressor.	Upregulation	(Hajra et al., 2002, Bolos et al., 2003)
Slug		E-cadherin repressor.	Upregulation	(Hajra et al., 2002, Bolos et al., 2003)
Twist		Cell lineage determination and differentiation.	Upregulation	(Yang et al., 2004, Maestro et al., 1999)
Zeb1		Transcriptional repressor of microRNA-200 family members and EMT inducer.	Upregulation	(Peinado et al., 2007, Eger et al., 2005, Burk et al., 2008)

Table 1.3. EMT biomarkers (epithelial and mesenchymal) and transcription factors characteristics as well as their expression during EMT. Every component of this complex network has different characteristics; some of which would enable cells to acquire mesenchymal features whilst losing epithelial properties. Their expression can be downregulated or upregulated, depending on their role in EMT.

1.10 Signalling pathways and EMT inducers

In cancer, EMT can be induced by extrinsic and intrinsic stimuli. The microenvironment of a tumour plays a very important role as an extrinsic stimulus during cancer progression and metastasis. Some EMT inducing factors can be promoted by the interaction between the microenvironment and tumour cells, for example, members of the Snail family (E-cadherin repressors) can be activated by extracellular factors. Extrinsic stimuli include activators of Wnt, Hedgehog, Notch, Nuclear Factor-κB (NF-κB) and TGF-β signalling pathways. Subsequently, TGF-β is a potent EMT inducer during tumour progression (Voulgari and Pintzas, 2009, reviewed by Huber et al., 2005 and Moustakas and Heldin, 2007). Crosstalk between these signalling pathways has been implicated in E-cadherin repression and EMT induction. For example, Wnt and Hedgehog signalling cascades induce Snail upregulation, which subsequently represses E-cadherin. Hedgehog signals interact with Notch and TGF-β signalling pathways to induce EMT (Katoh and Katoh, 2008). Wnt signalling pathway is disrupted during different cancer stages leading to EMT induction.

TGF-β is a member of a very complex multifunctional family of cytokines which act as mediators of development stages and tissue homeostasis. However, they have paradoxical functions depending on cell type. TGF-β can act as a physiological and pathophysiological regulator through different stages of life (reviewed by Singh and Settleman, 2010 and Derynck et al., 2001). For example; it can promote apoptosis and/or cell cycle arrest in normal epithelial, endothelial and haematopoietic cells, but when there is a deregulation or a mutation in the TGF-β pathway, apoptosis can be inhibited while cells acquire tumorigenic characteristics and also become motile and invasive to the surrounding tissues as reviewed by *Blobe et al., 2000*. Mutations in the TGF-β type II receptor were demonstrated by *Markowitz et al*., 1995 (Markowitz et al., 1995) and *Myeroff et al., 1995* (Myeroff et al., 1995) in different kinds of cancer showing loss of expression of this receptor. This resistance to its anti-proliferative characteristics can contribute to the formation of different kinds of cancer. The PI3K signalling pathway (induced by TGF-β), via its downstream effector Akt, can block apoptosis in mammary epithelial cells

chronically treated with TGF-β by sequestering Smad3 into the cytoplasm after insulin treatment (Gal et al., 2008, Remy et al., 2004). Moreover, TGF-β acts as a tumour suppressor during early stages of tumorigenesis by its growth inhibitory effects and stimulation of apoptosis. Interestingly, during late stages of tumour progression, it can also act as a tumour promoter with pro-metastatic effects. This was confirmed by *Shipitsin et al., 2007* (Shipitsin et al., 2007), who observed a high level of expression of TGF-β in CD44+/CD24- metastatic breast cancer stem cells. In addition, following TGF-β inhibition, these cells demonstrated a more epithelial behaviour. TGF-β can stimulate two of the most important features of cancer progression, which are invasion and metastasis (reviewed by Wendt et al., 2009). Furthermore, when TGF-β binds to its receptors (TGF-βRI and TGF-βRII), a signalling cascade is initiated by their phosphorylation, which can activate different proteins, in which the Smad family of transcription factors is one of the most notable. Specifically, when Smad2 and Smad3 bind with co-smad (Smad4) after their activation via TGF-βRI, this complex associates with different transcription factors like ZEB proteins to repress E-cadherin during EMT (reviewed by Derynck et al., 2001, Levy and Hill, 2005, Verschueren et al., 1999).

TGF-β also activates a range of downstream effectors, which can induce EMT. One of the most important downstream effector is NF-κB. As yet, the exact way NF-κB works is not fully understood, but one of its major roles is to suppress apoptosis. It remains unclear how NF-κB regulates EMT and metastasis, however, Huber et al, 2004 demonstrated that in Ras-transformed mammary epithelial cells, EMT is induced by gain of NF-κB (Huber et al., 2004). They also demonstrated that inactivation of NF-κB causes Mesenchymal to Epithelial Transition (MET), suggesting that this signalling pathway not only induces EMT, but also helps to maintain it. Inhibitory proteins of NF-κB, known as Inhibitor κB (IκB) proteins, which are involved in NF-κB activity regulation (Huber et al., 2004), have been utilised to further elucidate NF-κB function in different model systems. For example, *Huang et al., 2001* used a mutant form of IκBα to downregulate NF-κB activity in metastatic human prostate cancer and, as a result, they observed inhibition of tumour growth, invasion and metastasis *in vitro* and *in vivo* (Huang et al., 2001, Huber et al.,

2004). Furthermore, they also confirmed this in human melanoma cells (Huber et al., 2004, Huang et al., 2000). NF-κB regulates Snail expression, leading to its increased transcription. Subsequently, Snail represses Raf kinase inhibitor, an inhibitor of NF-κB activity. Therefore, Snail overexpression suppresses E-cadherin activity, inducing EMT while repressing RKI (Wu and Zhou, 2010).

Genetic alterations such as mutations/perturbations can act as intrinsic stimuli of EMT during cancer progression by potentiating EMT inducers or inhibiting EMT repressors. TGF-β can act as a tumour suppressor after acquiring a specific gene mutation, but its un-mutated form can function as an EMT inducer to promote invasion and metastasis (Levy and Hill, 2005). *Lehmann et al., 2000* showed that during cancer progression, the TGF-β growth inhibitory effect can have a negative feedback and cancer cells can proliferate due to an upregulation of Ras proteins and consequential activation of the MAP kinase pathway (Lehmann et al., 2000). TGF-β also lost its pro-apoptotic but not its pro-invasive activities through this pathway. TGF-β activates a range of different signalling pathways leading to EMT during tumour progression (Figure 1.4), such as Rho Family of GTPases (Figure 1.4 A), PI3K/AKT (Figure 1.4 B), Integrin-linked Kinase (ILK) (Figure 1.4 C), NF-κB (Figure 1.4 D) and MAP Kinases (Figure 1.4 E) (reviewed by Wendt et al., 2009). Each of these signalling pathways involves different features that are inter-related, for example, E-cadherin can be downregulated by a range of transcription factors, including Snail; Slug; Twist; and ZEB1 as reviewed by *Wendt et al., 2009*.

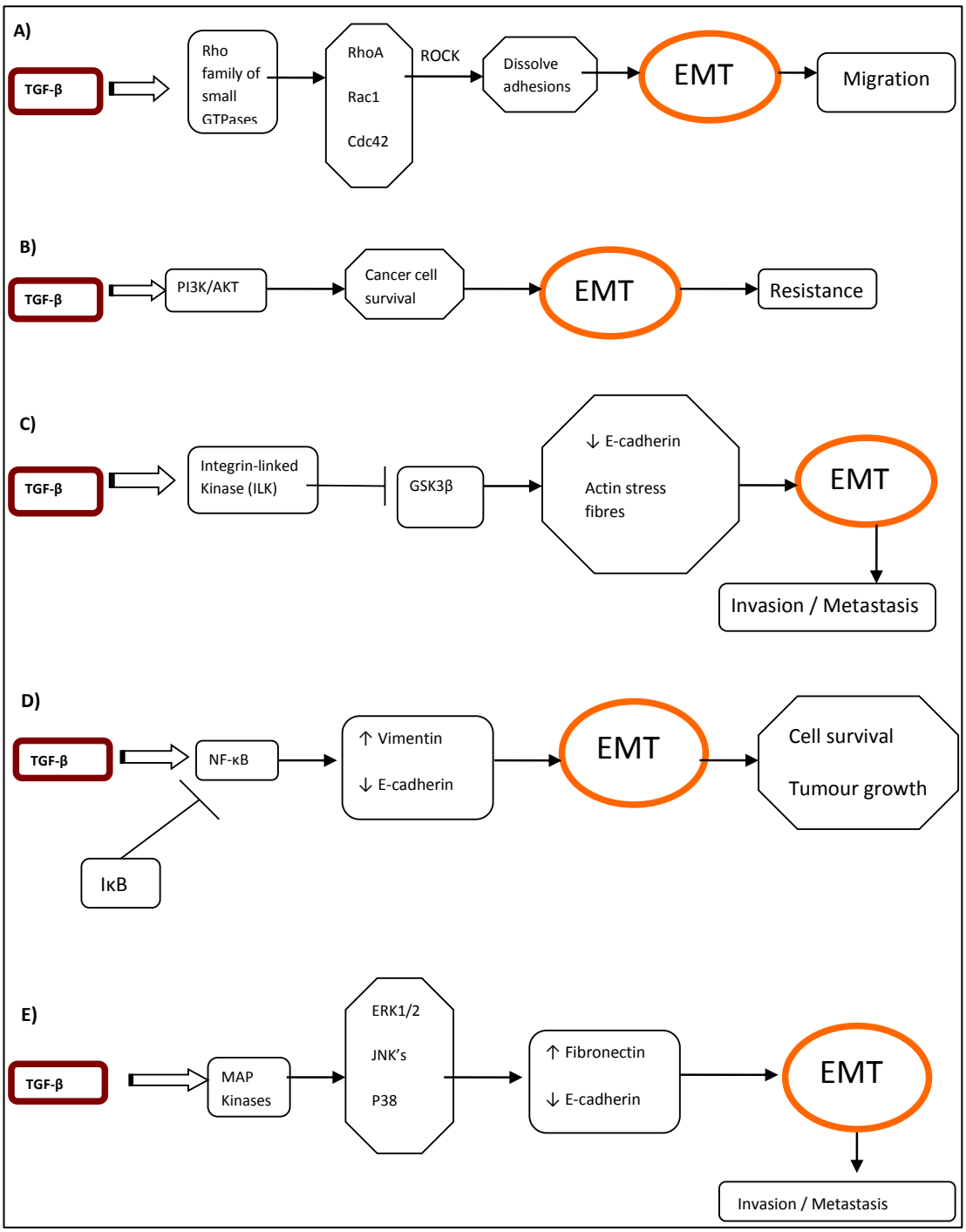

Figure 1.4. TGFβ1 stimulated signalling pathways. Among the signalling pathways stimulated by TGFβ1, there are at least these 5 that can promote EMT through different molecular changes. (A) Rho family of small GTPases. Comprised by RhoA, Rac1 and Cdc42. If RhoA is activated via TGF-β, epithelial cells can acquire mesenchymal characteristics by losing their cell-cell adhesion and gaining motility in order to migrate. This can be achieved by RhoA and its downstream effector ROCK. (B) PI3K/AKT. This signalling pathway is crucial to achieve EMT

Continued overleaf

because it enhances cancer cell survival. As we understand, when cells migrate through different tissues and vessels, they can be seriously damaged and killed. (C) Integrin-linked Kinase (ILK). Its elevated expression can be associated with downregulation of E-cadherin (cell-cell adhesions) and with formation of stress fibres and invasion which can be due to upregulation of fibronectin and vimentin. ILK can also be coupled with the AKT pathway. (D) NF-κB. This pathway can stimulate cell survival by regulating apoptosis, tumour cell growth and immune response. TGF-β suppresses its activity in normal cells, but it can stimulate it in cancer cells, leading to EMT by downregulation of E-cadherin and upregulation of vimentin. NF-κB can be repressed by IκB to block EMT and prevent migration. (E) MAP Kinases. This is a big family of protein kinases including ERK1/2, JNK's and p38 MAPKs. Activation of these family members by TGF-β promotes EMT by downregulating E-cadherin and increasing the formation of stress fibres with the upregulation of fibronectin. Invasion can also be achieved by the activation of prometastatic genes via p38 MAPKs.

1.11 Cancer Stem Cell Theory

The cancer stem cell theory proposes that a tumour contains heterogeneous cell populations. A small percentage of cancer cells have stem cell characteristics, such as self-renewal and pluripotency, enabling them to drive tumour recurrence and metastasis. These specific cells are able to produce all cell types within a tumour (reviewed by Nowell, 1976) and have been named cancer stem cells (CSCs) or tumour-initiating cells as reviewed by *Reya et al., 2001*. The concept that tumours can arise from small populations of tumorigenic cells was proposed in the late 19th century, but was tested almost one hundred years later by different research groups with human haematopoietic stem cells and leukaemia stem cells (Bonnet and Dick, 1997, Hope et al., 2004, reviewed by Dalerba et al., 2007, Eyler and Rich, 2008, and Winquist et al., 2009). These seminal studies helped to define this CSC theory by showing that a single cell was capable of generating a population of heterogeneous cells in mouse models. They confirmed that human acute myeloid leukaemia is arranged hierarchically, by looking for expression of haematopoietic cell-surface markers. They identified leukaemia-initiating cells as CD34+ CD38-, and capable of forming tumours in transplanted severe combined immune deficient (SCID) mice. In a similar study, *Lapidot et al., 1994* observed that CD34+ CD38- and CD34- cells did not have the same colony-forming properties (Lapidot et al., 1994).

Furthermore, research in different types of cancer is providing strong evidence of the existence of CSCs (reviewed by Reya et al., 2001). As previously explained, these tumour-initiating cells were first discovered in the haematopoietic system, but have been recently identified in several kinds of solid tumours including colon, breast and brain cancer (Al-Hajj et al., 2003, O'Brien et al., 2007, Ricci-Vitiani et al., 2007, Singh et al., 2004).

1.12 Linking EMT and Cancer Stem Cells

Recent evidence indicates that CSCs are required to establish metastatic tumours at distant sites *in vitro*, and that EMT may promote development of cancer cells with stem-cell characteristics and aid metastasis (reviewed by Reya et al., 2001, Mani et al., 2008).

In addition to morphological changes during EMT, epithelial cells may also develop altered functional properties, such as increased tumour-seeding ability, tumoursphere formation and expression of transcription factors Twist and Snail. In addition, they also play an important role in invasiveness and migration in different types of cancer as reviewed by *Gupta et al., 2009*. Independent groups have demonstrated *in vitro* that cells that have undergone EMT, by expressing different transcription factors or induced by TGF-β, showed stem cell characteristics, such as the ability to form spheres and expressed stem cell associated cell surface markers (Mani et al., 2008, Morel et al., 2008, and reviewed by Polyak and Weinberg, 2009). *Mani et al., 2008* showed that by over expression of Twist or Snail in immortalised human mammary epithelial cells (HMECs), these cells became more mesenchymal, and their expression pattern demonstrated downregulation of epithelial markers, such as E-cadherin, and upregulation of mesenchymal markers such as vimentin and fibronectin (Mani et al., 2008). After confirming that EMT took place in these cells, they used flow cytometry analysis to see if these cells had stem cell characteristics. This group also confirmed that these cells expressed CD44+CD24-, which are cell surface markers for mammary epithelial stem cells and human breast CSCs, and were capable of forming at least 30-fold more mammospheres than the untreated cells.

Cell sorting was performed in HMECs, mouse mammary stem cells and normal and neoplastic human breast stem cells and confirmed the overexpression of mRNAs encoding mesenchymal markers and downregulation of epithelial markers in CD44+CD24- (human) and CD49high CD24med (mouse). Immortalised HMECs, transformed by the HER2/neu oncogene, were then transfected with a vector expressing the tamoxifen-activatable form of Snail or Twist transcription factors in order to confirm the possibility of EMT generating cells with stem-like properties. These treated cells were assayed for tumoursphere forming efficiency where it was found that cells expressing Snail and/or Twist underwent EMT, and formed 10-fold tumourspheres than the untreated group (Mani et al., 2008). In a different study, EMT-derived cells were capable of differentiating into other cell lineages (Battula et al., 2010). *Morel et al., 2008* confirmed that CSCs can originate from a more differentiated cell line by utilising FACS analysis to demonstrate that cells expressing CD44+CD24-/low can be derived from cells expressing CD44lowCD24+ through the activation of the Ras/MAPK signalling pathway, and most interestingly, that this process can be stimulated and accelerated by EMT (Morel et al., 2008). The abilities of HMEC's and an oncogenic line (HMLER) to form mammospheres was compared and confirmed that only HMLER cells were able to form mammospheres. Subsequently the cell phenotypes of these different cell lines were analysed by FACS confirming that HMECs were CD44lowCD24+ and HMLER were CD44+CD24-/low. Furthermore, they performed cell sorting and single-cell cloning assays after H-RasV12 retroviral expression in HMECs and seeded CD24+ cells into 96-well plates with limiting cloning conditions and saw that after three weeks, 19% of the population of cells were CD44+CD24-. This population of cells was able to grow tumours when injected into mammary pads of nude mice, compared with CD44lowCD24+, which were not able to establish tumour growth. CD44+CD24- cells in HMEC and MCF10 (immortal human mammary epithelial cell line) cell lines showed a spindle shape and expressed lower levels of E-cadherin and β-catenin (epithelial markers) and higher levels of fibronectin and vimentin (mesenchymal markers), suggesting that these stem cell properties were related to EMT. Finally it was confirmed that EMT enabled cells to acquire stemness when the CD44-CD24+ cell lines were treated with TGFβ1, which is one of the most potent EMT inducers,

for eight days and showed upregulation of vimentin and downregulation of E-cadherin, as well as presence of CD44+CD24- cells (Morel et al., 2008). Collectively, these results strongly suggest that EMT is potentially a precursor to generate CSCs from more differentiated cell lines. Further work is required to consolidate these theories.

1.13 Clinical and therapeutic implications of an EMT/CSC Axis

EMT may play a fundamental role in cancer progression and metastasis, thus, the development of novel therapeutic and diagnostic protocols targeting this transition process would be relevant in the search for cancer treatment. EMT has been associated with resistance to antineoplastic agents (Voulgari and Pintzas, 2009), and if this feature is confirmed, further research could lead to new treatment against cancer progression and metastasis.

Recent data demonstrates that epithelial cells induced by EMT may play an important role in invasiveness and migration in different types of cancer as shown in Figure 1.3 (reviewed by Gupta et al., 2009). The percentage of CSCs in a tumour may vary between different tumours and patients (Figure 1.5 A), as reviewed by Pardal et al., 2003. The subpopulation of CSCs identified in human breast cancer exhibit CD44+ and CD24- cell surface-markers, and were found to be more resistant to conventional therapies than the more differentiated cancer cells, (Li et al., 2008) suggesting that tumour relapse can be accomplished by these CSCs after treatment (Figure 1.5 B). Therefore, these cells must be eliminated to affect a cure on cancer (Figure 1.5 C).

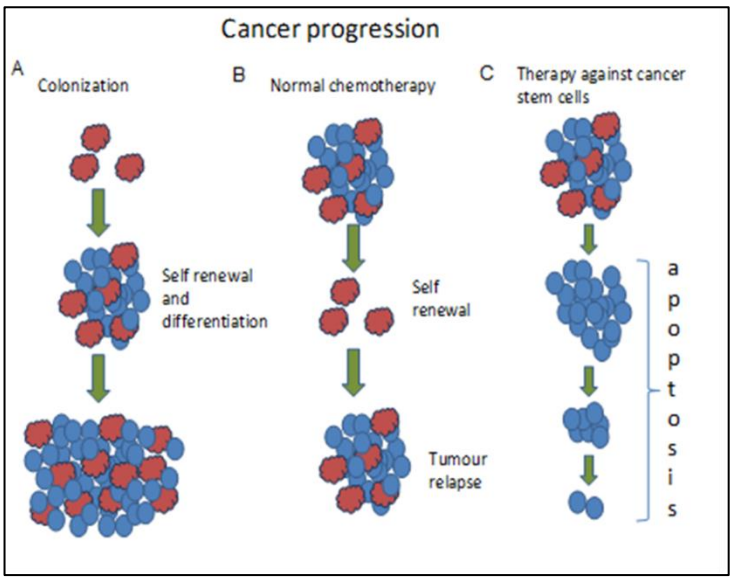

Figure 1.5. Cancer stem cells are resistant to conventional therapy. A) Self renewal and differentiation from single cancer stem cells (red) to differentiated cancer cells or progeny (blue). B) Tumour relapse after conventional chemotherapy against differentiated cancer cells. C) If cancer stem cells are eliminated, then their progeny would pass through a period of multiple apoptosis, and the result would be a slowly decrease in tumour cells until the tumour disappears completely.

As CSCs are proposed to be more resistant to conventional therapies, it becomes necessary to find new ways to target them in order to treat cancer and prevent its recurrence. The central role that EMT has in tumour progression makes it an obvious target for therapeutic intervention. *Olmeda et al., 2007a* have shown that by silencing Snail with shRNA, they can block EMT by enabling E-cadherin expression and leading to a MET process (Krutzfeldt et al., 2005, Olmeda et al., 2007a). EMT plays a main role in generating CSCs, screening with different drugs to block EMT and its precursors (transcription factors) may inhibit cancer progression.

If this crosslink between EMT and CSCs is confirmed, treatment of cancer at early stages should include EMT blocking agents that could inhibit TGF-β downstream effectors like ZEB1. By these means, resistance to apoptosis and self-renewal characteristics might be nullified, preventing not only migration of cancer cells, but also the development of the primary tumour by stopping CSCs multiplication (Figure 1.6). However, the inhibition of EMT may also have serious

consequences for wound healing processes and tissue remodelling during repair and regeneration.

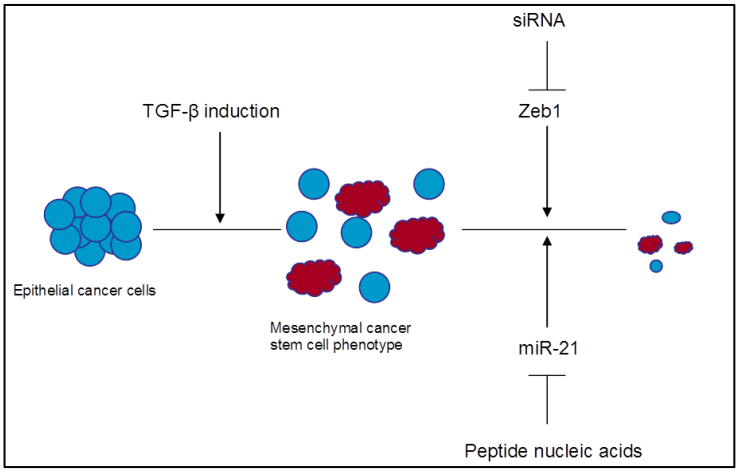

Figure 1.6. Therapeutic intervention for EMT. This diagram shows two different possible therapeutic levels regarding the blockage of EMT precursors and miR-21 as an oncomir. After inhibiting Zeb1 and/or miR-21 cancer stem cells would lose their acquired stem cell characteristics and would start dying by apoptosis and stop dividing.

1.14 Cellular Reprogramming, EMT and E-Cadherin

Cell reprogramming can be defined as the ability of specific transcription factors to change the identity of specialised differentiated cells into different cell lineages (Graf and Enver, 2009, and reviewed by Sanges and Cosma, 2010). As described by Yamanaka et al., 2006, it is an important step in the acquisition and maintenance of pluripotency of epithelial cells *in vitro* (Figure 1.7) achieved by defined pluripotency transcription factors: OCT4, SOX2, KLF4 and c-MYC (OSKM), also known as the Yamanaka cocktail (reviewed by Lowry, 2011, Takahashi and Yamanaka, 2006). This group showed that pluripotency can be induced *in vitro* in mouse embryonic fibroblasts (MEFs), and these can be reprogrammed by the above mentioned factors to become induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006).

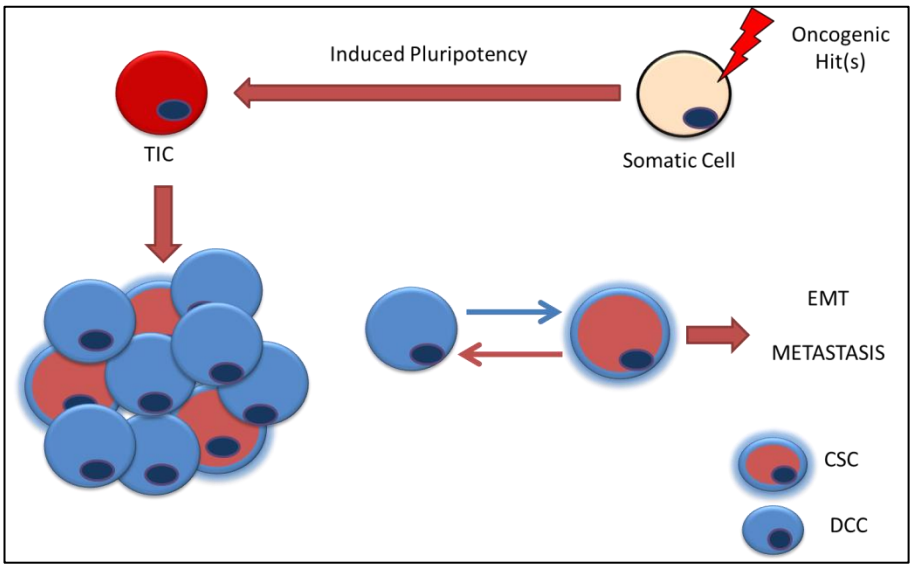

Figure 1.7. Cell reprogramming and cancer stem cells. A somatic cell can be induced by oncogenic mutations to acquire pluripotency and become a tumour initiating cell (TIC), which will generate cancer stem cells (CSC) and differentiated cancer cells (DCC), and might lead to metastasis after an EMT process.

In a review paper published by *Sanges and Cosma, 2010* it has been described that a MET is necessary for the reprogramming process (Sanges and Cosma, 2010), which was then confirmed by *Redmer et al., 2011*, showing that E-cadherin might play an important role in maintaining pluripotency in mouse

embryonic stem cells (mESCs), and it can also replace OCT4 during reprogramming with a MET (Redmer et al., 2011). How E-cadherin maintains pluripotency was not further explored in that study. However, its role was confirmed by replacing OCT4 in the OSKM combination with an E-cadherin-expressing retrovirus which demonstrated that this new combination of factors reprogrammed MEFs into iPSCs. These iPSCs were able to form teratomas in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, while clones lacking OCT4 and E-cadherin expression just formed small lumps (Redmer et al., 2011).

The metastatic progression of different types of cancer is carried out by a complex network of pathways in which E-cadherin plays different roles in EMT, MET and as mentioned above, cell reprogramming (Figure 1.8). The role of E-cadherin in cell reprogramming as a pluripotency inducer could be one of the essential features of metastasis due to its ability to change cellular identity once the cancer cells have reached their new niche after EMT and subsequent MET (in which E-cadherin is highly upregulated).

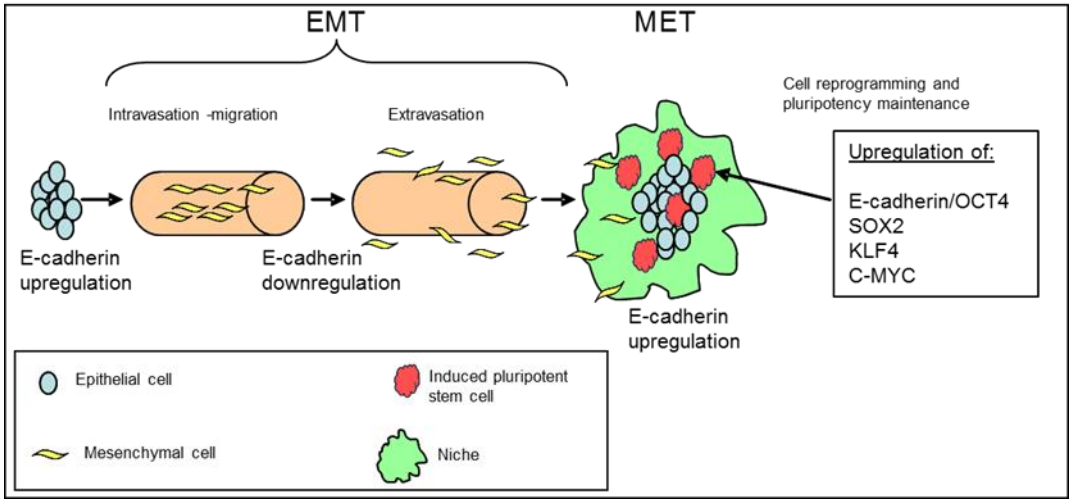

Figure 1.8. Cell reprogramming. After epithelial cells acquire mesenchymal characteristics by downregulation of E-cadherin during EMT and migrate through blood and lymphatic vessels, they colonise their new target organ (niche) when E-cadherin is upregulated and cells return to their epithelial phenotype through a MET. While these cells colonise their new niche, E-cadherin and defined pluripotency factors (OCT4, SOX2, KLF4 and c-MYC) reprogram cells to become pluripotent stem cells.

Furthermore, *Liao et al., 2011* showed that cell reprogramming through a MET could also be carried out by microRNA (miRNA) expression in mesenchymal cells. They demonstrated that overexpression of miR-302 complex in addition to miR-367 in fibroblasts was enough to downregulate TGF-β receptor 2, thus, facilitating MET by upregulation of E-cadherin and cell reprogramming into iPSCs (Liao et al., 2011). These findings support a link between the epithelial phenotype and pluripotency, suggesting potential targets for prevention or treatment of metastases.

1.15 microRNA expression in cancer

MicroRNAs (miRNAs) are small RNA molecules with 19-25 nucleotides, which can work in a post-transcriptional fashion (reviewed by Ambros, 2004 and Hwang and Mendell, 2006) and contribute to fundamental physiological cell processes, such as tissue differentiation, and in the progression of several diseases like cancer (Foubert et al., 2010). MiRNAs guide the RNA-induced silencing complex (RISC), targeting complementary mRNAs, but they can also be targeted by other mRNAs as reviewed by *Bartel, 2004*. Targeted mRNAs are usually translationally silenced or cleaved by RISC, depending on its degree of complementarity with the targeting miRNA. When there is an imperfect complementarity between an miRNA and an mRNA, the target is translationally silenced, a mechanism which is activated by most mammalian miRNAs. However if there exists a perfect complementarity between miRNA and target mRNA, cleavage by RISC follows (reviewed by Hwang and Mendell, 2006). Bioinformatic assays have suggested that miRNAs are able to regulate approximately 30% of all genes of a mammalian genome (Lewis et al., 2005). Interestingly, recent data shows that more than 50% of predicted human miRNAs are located within fragile genomic sites susceptible to mutations during oncogenesis and tumour progression (Calin et al., 2004 and reviewed by Croce, 2009).

The knowledge that some cancer-related genes can be repressed or promoted by miRNAs is useful in the rational development of diagnostics and therapeutics.

Some miRNAs repress tumour promoter genes while others are able to repress tumour suppressor genes by inhibiting target genes encoding oncogenic or tumour suppressor proteins as reviewed by Croce, 2009. MicroRNAs promoting cancer and its progression have been described as oncomirs (Taylor and Schiemann, 2014). *Yan et al., 2011* inhibited human breast cancer proliferation *in vitro* and *in vivo* by blocking microRNA-21 (miR-21) in MCF7 cells. After confirming that these cells overexpress miR-21, they utilised sequence-specific functional inhibition of miR-21 by transfection with a locked nucleic acid-antimiR-21, observing inhibition of cell growth, proliferation and migration *in vitro*. Moreover, in order to confirm these effects *in vivo*, MCF7 cells treated with peptide nucleic acid-antimiR-21 before injection were injected subcutaneously into female nude mice in which the detectable tumour masses were fewer and smaller than those induced by control cells (Yan et al., 2011). The inhibition of oncomirs might be a useful resource to block EMT-induced cancer progression. There is still much to be elucidated on how these gene regulators are involved in cancer and its progression. There are different families of identified miRNAs which can regulate different functions in the organism and the cell cycle, including differentiation and apoptosis, but it is thought that cancer and its progression might also be a consequence of an impaired miRNA expression, either inhibiting tumour suppressor genes or enhancing tumour promoter genes as reviewed by Esquela-Kerscher and Slack, 2006. Further investigation is needed to confirm the role of miRNAs in cancer. The identification of diverse miRNAs expression in different kinds of tumours might be a useful tool for diagnostic and therapeutic approaches in cancer and its progression.

MicroRNAs have also been involved in several studies of EMT and CSC theory. *Gregory et al., 2008* observed that after suppression of endogenous miR-200 family members, EMT was induced in normal and cancer cell lines through ZEB1 and ZEB2 targeting (Gregory et al., 2008). Moreover, another study showed that after chemotherapy exposure, resistant breast cancer cells did not express members of the miR-200 family, suggesting that these stem-like, chemotherapy-resistant cancer cells lack expression of the miR-200 family, in contrast with the chemotherapy-sensitive cancer cells from the same patients (Yu et al., 2007).

There is still much to be understood and further investigation is needed to fully elucidate the role of miRNAs in cancer and its progression.

1.16 EMT in companion animals

EMT in companion animals (dogs and cats) has not been widely studied. *Chandler et al., 2007* showed the importance of Slug as a transcription factor during cell migration in wound healing. They induced corneal wounds in 12 dogs and measured Slug expression in wounded and unwounded corneas in order to assess the rate of wound healing with and without Slug expression. Their results showed an upregulation of Slug expression in wounded corneas compared to the unwounded ones. Interestingly, downregulation of E-cadherin and β-catenin (epithelial markers) was observed in healing tissues, meaning that cells were losing their adherens junctions, consistent with enhanced ability to move through the cornea (Chandler et al., 2007).

Furthermore, in two different studies, *Aresu et al., 2008 and 2007*, the role of EMT in canine renal fibrosis was studied, in which EMT was found to be essential to generate this disease. During renal inflammation-fibrosis, leading to diseases such as glomerulonephritis, epithelial markers E-cadherin and β-catenin (Aresu et al., 2008), and cytokeratin (Aresu et al., 2007) are downregulated whilst the mesenchymal marker vimentin is upregulated at sites of inflammation. Similarly, a study conducted in 2009 by *Mathias et al*. highlighted the involvement of several proteins during EMT in Madin-Darby canine kidney (MDCK) cells. They compared the secretome from MDCK and oncogenic Ras-transformed MDCK cells using a combination of fluorescence difference gel electrophoresis labelling and mRNA transcript analysis in order to identify proteins that are differentially expressed during EMT in these cells. They first showed that Ras induces EMT in MDCK cells where Ras-transformed cells morphologically appeared more fibroblast-like with an elongated shape, while untransformed MDCK cells appear round and tightly adjoined with surrounding cells. They also observed a lower expression of the epithelial marker E-cadherin and a higher expression of the mesenchymal marker vimentin in Ras-transformed cells. To assess whether the Ras-transformed cells acquired migratory features, a wound

healing assay was conducted in which untransformed MDCK cells exhibited a limited migratory capacity. In contrast, Ras-transformed MDCK cells showed a more dynamic migration as individual cells moved faster into the wounded area. They demonstrated that cells undergoing EMT increased secretion of proteases that facilitate migration, such as MMP-1, and also decrease expression of proteins important in cell-cell and cell-matrix adhesion such as clusterin and desmocollin (Mathias et al., 2009). Interestingly, some of these proteins had not been previously described as important components of the mechanisms underlying EMT.

Several research groups have published important results regarding type II EMT (wound healing and fibrosis), but these studies have yet to be applied to type III EMT (cancer progression and metastasis).

Several authors from different research groups have published interesting results regarding expression of cell adhesion molecules and their correlation with tumour growth and cell proliferation. *Han et al., 2010* observed an increased expression of β-catenin accumulation in the cytoplasm and nucleus in canine cutaneous melanotic tumours (Han et al., 2010). Normally, β-catenin is degraded in the cytoplasm; however, deregulation of the Wnt/β-catenin signalling pathway leads to accumulation of this protein, causing uncontrolled cell proliferation (reviewed by Larue and Delmas, 2006). These findings have been also documented in canine colorectal tumours (McEntee and Brenneman, 1999) and canine osteosarcoma (Stein et al., 2011). Moreover, *Nowak et al., 2007 and 2008* compared the relationships between extracellular matrix metalloproteinase-9 (MMP-9), E-cadherin, the proliferation associated antigen Ki-67 and β-catenin in canine mammary adenocarcinoma, and found that the decreased expression of E-cadherin is inversely proportional to the expression of MMP-9, Ki-67 and nuclear-located β-catenin, whereas a direct correlation was observed between MMP-9 and Ki-67; and β-catenin and Ki-67 expression. They also observed increased cell growth and proliferation associated with higher expression of nuclear accumulation of β-catenin (Nowak et al., 2007, Nowak et al., 2008).

In canine colorectal adenocarcinoma, *Aresu et al., 2010* observed lower expressions of E-cadherin and β-catenin on the cell membrane of the majority of analysed tumours. A correlation between low expression of E-cadherin with higher grade tumours and higher mean age of patients was observed in this study. Reduced expression of β-catenin was also related to tumours with higher grade, and with increased tumour size (Aresu et al., 2010). Furthermore, *Ide et al., 2011* found that increased expression of cell adhesion molecules N-cadherin, doublecortin and nuclear β-catenin was closely associated with progression of canine meningioma (Ide et al., 2011).

Furthermore, to assess the correlation between adhesion molecules and transcription factors in cancer progression, *Im et al., 2012* examined the role of Snail in canine mammary tumours. Even though they did not observe any association between the expression of Snail and E-cadherin in mammary tumours (11 adenomas and 43 carcinomas), they identified a significant correlation of Snail expression with tumour grade, histological type and lymphatic invasion, where they observed higher expression of this transcription factor in carcinoma samples when compared with adenomas and normal tissues (Im et al., 2012).

A significant paper was published in 2014 by Salgado et al., where they assessed the expression of EMT-associated proteins by immunohistochemistry in canine mammary carcinoma. They observed loss of epithelial marker E-cadherin and overexpression of mesenchymal proteins (N-cadherin, Snail, S100A4 and MMP-2) in carcinomas, but not in benign tumours or non-neoplastic lesions. High expression of Snail was related to loss of E-cadherin. They suggested that these findings might have great importance to evaluate canine mammary carcinoma patients, as they could serve as an important tool to distinguish between adenomas and carcinomas (Salgado et al., 2014). On a similar approach, Fonseca-Alves et al., 2015, carried out a study on type III EMT in prostatic carcinoma in dogs. One of the most important findings they showed was loss of E-cadherin and a higher expression of vimentin at the protein expression level in the tumour group compared with normal tissue samples. They also carried out immunohistochemical analyses where they found loss of

epithelial markers and overexpression of mesenchymal markers in metastatic prostate carcinoma cells (Fonseca-Alves et al., 2015).

1.17 Aims of our research

The principal aim of this research was to determine if induction of EMT can generate cells with a CSC phenotype. To study this we induced EMT in a panel of mammary carcinoma cell lines by treating them with TGF-β, and subsequently analysed expression of EMT markers and sphere forming ability. Our hypothesis was that canine and feline mammary cancer cells undergo phenotypic changes after TGF-β-induced EMT, acquiring stem cell characteristics and invasive properties.

Here, we have studied EMT as a feature of human, canine and feline mammary carcinoma in an attempt to elucidate the role of EMT in cancer invasiveness and in the acquisition of stem cell-like characteristics. We have demonstrated that dog and cat cells undergoing EMT by TGF-β stimulation show a mesenchymal morphology; increased expression of mesenchymal markers (fibronectin and vimentin); decreased expression of epithelial markers (E-cadherin and β-catenin) and increased cell invasion capacity *in vitro*. Interestingly, cells that underwent EMT were more capable of forming spheres, which are representative of CSCs. We also carried out miRNA expression analysis in TGF-β-stimulated canine mammary carcinoma cells and untreated cells. We found significant differences in specific canine miRNAs suggesting their importance in the study of mammary tumours in dogs. Although further investigation is needed, such as the correlation of our *in vitro* findings in clinical samples, these results suggest EMT is a worthwhile potential therapeutic target in companion animal oncology.

In conclusion, EMT is an emerging field of cancer research. This transition process might be very relevant to cancer induction and progression. Moreover, if we can confirm that the EMT/MET process enables cancer cells to acquire stem-cell characteristics, then we would be able to generate new targeted therapies against cancer and its progression and metastasis, potentially combatting tumour relapse and metastasis in thousands of patients around the world.

Chapter 2: Material and Methods

2.1 Tissue culture reagents and equipment

Tissue culture plastics were obtained from Becton Dickinson-Biosciences, UK (Falcon tubes) and Sigma-Aldrich, UK (Corning® low adhesion tissue culture plates and pipette tips).

The microscope used for all fluorescence and phase contrast images was a Zeiss Axiovert40 CFL microscope fitted with digital Axiocam monochrome or colour cameras (Carl Zeiss Ltd., UK). Carl Zeiss Imaging systems AxioVisionTM Software (Release 4.27.2) was used for image capture and image processing.

2.2 Cell Culture

2.2.1 Cell lines

The MCF7 cell line is a fully characterised human breast carcinoma cell line and was a kind gift from Dr. Maura Wallace at the Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh.

The REM cell line is a canine mammary carcinoma cell line derived from a spontaneous primary canine mammary carcinoma. The CAT M cell line (CMC) is a feline mammary carcinoma cell line derived from a feline mammary adenocarcinoma. These cell lines were received from Professor Rod W. Else, Royal (Dick) School of Veterinary Studies, University of Edinburgh (Else et al., 1982, Norval et al., 1985).

All cell culture reagents were obtained from Gibco® Invitrogen, UK unless otherwise specified. The MCF7, REM and CMC cell lines were grown in Dulbecco´s Modified Eagle´s Medium (DMEM) 41965 supplemented with 10% FBS, 1% Penicillin-Streptomycin at 37°C and 5% CO_2.

2.2.2 Cell Recovery

To recover cells from a frozen stock at – 70 °C, cells were quickly thawed at 37 °C and resuspended in 5 ml phosphate buffered saline (PBS) and centrifuged at 125 x g for 5 minutes. The supernatant was discarded and the cell pellets were resuspended in 8 ml of their respective media, transferred to T25 flasks, and incubated at 37 °C and 5% CO_2 overnight.

2.2.3 Passaging cells

To detach adherent cells, 0.25 % trypsin-EDTA (Life Technologies, USA) was used. At first passage, cell lines were transferred to T75 flasks. Cells were routinely passaged at 80-95 % confluency every 3-4 days. To passage cells, media was removed by pipetting, and the cells were washed twice in 10 ml of PBS before incubation at 37 °C in 3 ml of 0.25 % trypsin-EDTA until detached. Trypsinisation was terminated by adding at least twice the trypsin volume of FBS containing media to the cells. After counting in a haemocytometer, 3 x 10^6 cells were seeded directly into new T-75 (75 cm^2) culture vessels with fresh medium.

2.2.4 Determining cell density and viability

Cell counts were performed with a haemocytometer (Neubauer ruling: Square volume 1x10-4 ml; grid volume 9x10-4 ml) (Sigma-Aldrich). Ten microliters of cell suspension mixed with 10 µl of 0.4 % trypan blue (Sigma-Aldrich) was pipetted into each side of the counting chamber. Cells excluding trypan blue were counted within at least 4 squares. The result was divided per each square counted in order to get the average count for each side of the chamber. The result was then multiplied by 2, due to the dilution factor after adding trypan blue, so the cell count was calculated as:

Number of cells = (Average count x 2) x 104 cells per ml.

2.2.5 Harvesting adherent cells

To harvest cells, medium was discarded and cells were washed with ice-cold PBS, then scraped in 1 ml ice-cold PBS and transferred to 1.5 ml eppendorf tubes (Becton Dickinson-Biosciences). The cell suspension was centrifuged at 200 x g for

4 minutes at 4 °C. Supernatant was discarded and cell pellets were snap frozen on dry ice and stored at -70 °C.

2.2.6 Cryopreservation

Cells were frozen in freezing media containing 10 % Dimethyl Sulphoxide in FBS (Sigma-Aldrich). After washing and trypsinisation, cell suspensions were centrifuged at 125 x g for 5 minutes and resuspended in 1 ml of freezing medium, aliquoted into cryo-vials (Thermo-Scientific, USA), transferred to freezing container containing 100 % isopropyl alcohol (Mr Frosty, Thermo.Scientific) and placed in a -80 °C freezer for a minimum of 3 hours. Using a freezing vehicle allowed for a steady freezing rate of 1 °C per minute which enhances successful cryopreservation of cells and decreases overall cell death (Simione, 1992) . Cells were later transferred to liquid nitrogen for long term storage.

2.3 TGF-β

TGF-B was purchased from PeproTech EC Ltd., UK and reconstituted as indicated by the manufacturer. Briefly, the vial was centrifuged prior to opening and reconstituted in 10 mM Citric Acid (Sigma-Aldrich), pH 3.0 to a concentration of 0.5 mg/ml. it was further diluted in 0.1 % BSA (Sigma-Aldrich) and stored in working aliquots of 60 µl at -20 ºC.

2.3.1 TGF-β treatment

Cells were treated at the indicated concentration (2.5 – 10 ng/ml) at different time points. After observing morphological changes after TGF-β stimulation, control, mock-treated and TGF-β-stimulated cells were harvested according to Chapter 2.2.5 and stored at -70 ºC. Each sample was harvested in two eppendorf tubes that then were used for either protein or RNA extraction. For protein expression, the samples were harvested after 6 days of TGF-β stimulation. For mRNA expression analysis by qRT-PCR, samples were harvested at different time points for 24 days with different concentrations of TGF-β (2.5, 5 and 10 ng/ml).

2.3.2 Time course

TGF-β stimulation was optimised by treating cells with different concentrations of TGF-β. The concentrations utilised for TGF-β treatment were 2.5, 5 and 10 ng/ml of medium (previously enriched with FBS and antibiotics) in the three different cell lines. Medium was changed and TGF-β was added to cells every day. Cells were passaged as outlined in chapter 2.2.3, when approaching confluency, and seeded with medium enriched with TGF-β. In order to assess which concentration of TGF-β induced the greatest morphological change and to define how long epithelial breast cancer cells needed such TGF-β stimulation to induce morphological changes, we treated cells at different time points with the three different concentrations previously mentioned.

2.4 Sphere assay protocol

2.4.1 Setting up spheres from adherent cells

For sphere assays the cells were grown in N2 media with methylcellulose prepared in house. All the constituents for the N2 media were obtained from Sigma-Aldrich, except the recombinant human epidermal growth factor (EGF) and the recombinant human basic fibroblast growth factor (bFGF) which were obtained from PeproTech EC Ltd. The N2 media was prepared in a laminar hood using basic protective clothing. To 15.6 g of double standard strength DMEM/F12 base medium, was added 474 ml of distilled water, 12.5 mg transferrin, 5 µg EGF and 5 µg bFGF. The resultant solution was made up to 20 nM progesterone, 100 µM putrescine, 30 nM sodium selenite and 3.48 µM insulin, making a final volume of 500 ml. An equal volume of 1.6% methylcellulose dissolved in distilled water which had been autoclaved was added to a final volume of 1000 ml. The pH of the DMEM/F12 medium was adjusted to 7.2 utilising sodium hydroxide (NaOH) or hydrochloric acid (HCl) as needed before the solution was filtered sterilised through a 0.22 µm filter bottle (Corning® 500mL Vacuum Filter/Storage Bottle System. USA). The resulting N2 medium was aliquoted into 100 ml sterile bottles and stored at -20°C until use. Thawed media stored at 4°C were used for up to 4 weeks.

REM and CMC cells were trypsinated as described in Chapter 2.2.3, and 1 ml of cell suspension was removed and centrifuged at 100 x g for 5 minutes and resuspended in 1 ml PBS. Cells were then counted and seeded in six-well low adherence plates (Sigma-Aldrich). Per well, 60,000 cells were seeded in 2 ml of N2 media. Cells were incubated at 37 °C/5 % CO2 and N2 media was supplemented with 500 µg per ml of EGF and bFGF each every 48 hours.

2.4.2 Passaging spheres

To passage spheres, cells were transferred to Falcon tubes and the wells were washed in PBS to maximise collection. The pooled cells and washings were then centrifuged at 125 x g for 5 minutes and the N2 media was discarded. Five ml of 0.25% trypsin-EDTA was added to the cells and incubated at room temperature for 10 minutes. Three ml of media containing FBS was added to terminate trypsinisation. Cells were collected by centrifugation at 125 x g for 5 minutes. The cell pellet was resuspended in 1 ml of PBS and counted using a haemocytometer as outlined in chapter 2.2.4. A six-well low adherence plate was seeded with 60,000 cells per well in 2 ml of N2 media.

2.5 Recovery and detection of protein

2.5.1 Reagents and antibodies

All samples were probed with primary antibodies against EMT markers. The primary antibodies used were mouse monoclonal anti-E-Cadherin, clone 36/E-cadherin (BD Biosciences, UK) at a 1:5,000 dilution, mouse monoclonal anti-β-Catenin, clone 14/Beta-catenin (BD Biosciences) at 1:2,000 dilution, mouse monoclonal anti-fibronectin, clone 10/Fibronetin (BD Biosciences,) at 1:10,000 dilution, mouse monoclonal anti-vimentin, clone RV202 (Abcam, UK) at 1:1,000 dilution, rabbit polyclonal anti-Twist (L-21) (Santa Cruz Biotechnology, USA) at 1:100 dilution, rabbit polyclonal anti-ZEB1 (H-102) (Santa Cruz Biotechnology) and mouse monoclonal anti-β-actin (Abcam) at 1:4,000 dilution. The secondary antibodies used were polyclonal swine anti-rabbit (SAR) HRP conjugated

immunoglobulins (P0217) and polyclonal rabbit anti-mouse (RAM) HRP conjugated immunoglobulins (P0260), both purchased from Dako Cytomation, Denmark.

2.5.2 Cell lysis

All manipulations were performed on ice. Cells were harvested as described in chapter 2.1.5. Twice the pellet volume of chilled urea lysis buffer (7M urea (Sigma-Aldrich), 0.1M DTT (Sigma-Aldrich), 0.05% Triton X-100 (Sigma-Aldrich), 20mM HEPES-KOH (Life Technologies), pH7.6, 25mM NaCl (Sigma-Aldrich)) was added to the frozen cell pellet, and allowed to thaw on ice. The pellet was then mixed by pipetting until no cell clumps were visible and incubated for 30 minutes on ice. The cell lysates were centrifuged at 4°C at 1000 x g for 10 minutes, snap frozen on dry ice, and stored at minus 70°C.

2.5.3 Bradford assay

One µl of cell lysate or known BSA protein standard dilutions was mixed with 200 µl of Quick Start™ Bradford Dye Reagent (BioRad, USA) and added to a clear 96 well plate (Sigma-Aldrich). The absorbance at 595 nm was measured with a Perkin Elmer 1420 Multilabel Counter Victor3™ plate reader (Perkin Elmer, USA) and the protein concentrations of the different cell line lysates were determined from a standard curve generated from the known BSA concentrations using Excel, Microsoft Office, version 97-2003. This information was used to standardise the amount of lysate protein to be used for gel loading for Western Blot analysis.

2.5.4 SDS polyacrylamide gel electrophoresis (SDS PAGE)

Protein samples were resolved on denaturing SDS-polyacrylamide gels by electrophoresis. Different percentage SDS-polyacrylamide gels were poured according to size of protein of interest with low percentage gels used to separate high molecular weight proteins (e.g. fibronectin: 240 kDa) and higher percentage gels for lower molecular weight proteins (e.g. Twist: 24-41 kDa).

The SDS-polyacrylamide gels were prepared and assembled using the Protean II minigel system (Bio-Rad). The resolving gel (8-10 % acrylamide) was made according to manufacturer´s instructions and was poured and overlaid with

isopropanol to remove air bubbles. The gel was allowed to set at room temperature for 20 minutes, and the isopropanol was removed. The stacking gel (5 % acrylamide) was made according to manufacturer´s instructions and was poured, ten well loading combs were inserted, and the gel was allowed to set at room temperature for 20 minutes.

Protein samples were prepared into 45 µl aliquots in 4 x Laemmli Sample buffer (120 mM Tris-HCl (Sigma-Aldrich) pH 6.8, 4 % (w/v) sodium dodecyl sulphate (SDS) (Sigma-Aldrich), 20 % (v/v) glycerol (Sigma-Aldrich), 200 mM DTT (Sigma-Aldrich), 0.04 % (w/v) bromophenol blue (Sigma-Aldrich)) and boiled in a heat block for 5 minutes at 95 °C to denature the proteins. The gel was immersed in running buffer (25 mM Tris (Sigma-Aldrich), 190 mM glycine (Sigma-Aldrich), 0.1 % (v/w) SDS (Sigma-Aldrich)). Each well was loaded with 10-30 µg of protein lysate diluted in Laemmli Sample (10 µl total volume) and the gels were run at 180 V for approximately one hour using the Mini-protean electrophoresis system (Bio-Rad), until the dye had run off the resolving gel. Five µl of pre stained Full Range Rainbow Marker (Amersham Pharmacia Biotech, Sweden) was run in parallel with the protein samples to allow size determination of protein bands.

2.5.5 Immunoblotting

The resolved proteins were electrophoretically transferred using the Mini-protean electrophoresis system (Bio-Rad), onto nitrocellulose membranes (Hybond™-C, Amersham Biosciences, UK) in transfer buffer (25 mM Tris (Sigma-Aldrich), 190 mM glycine (Sigma-Aldrich), 20 % (v/v) methanol (Sigma-Aldrich)) at 300 mA for 60 minutes or 30 mA overnight. Nitrocellulose membranes were ink stained (0.4 % ink (Pelikan, UK) in PBS) for 15 minutes to visualise protein bands and ensure equal loading. The membranes were blocked for 1 hour at room temperature in PBST/5 % Milk (5 % Skimmed milk (Sigma-Aldrich) in 2% PBS-tween 20 (Sigma-Aldrich)) before being incubated with primary antibodies for 4 hours at room temperature or overnight at 4 °C in blocking solution.

After three 5 minutes washes in 2% PBST the membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibodies diluted

1:1,000 in blocking solution. After a further three 15 minutes washes with 2% PBST the blots were developed using ECL Western Blotting Detection Reagents (Amersham Biosciences) and sites of peroxidase activity were visualised by exposure to X-ray film (Hybond™-ECL™ Film, Amersham Biosciences).

2.6 Isolation and quantification of nucleic acids

2.6.1 RNA extraction

Total RNA was isolated from the cells utilising two methods: The RNeasy Mini Kit with QIAshredder (Qiagen Ltd., UK) according to the manufacturer's Animal Cells Spin protocol including a DNase on-column digestion step utilising the RNase-free DNase Set (Qiagen); and the RNA Bee protocol (AMS Biotechnology, UK), according to the manufacturer's protocol, adding chloroform, isopropanol and ethanol. All samples were analysed with NanoDrop™ ND1000 (Thermo Fischer Scientific, USA) to quantify RNA.

2.6.1.1 RNeasy Mini Kit with QIAshredder:

This commercial kit extracts RNA from cells utilising the spin column-based nucleic acid extraction principle. Cells were counted and lysed by adding 350-600 µl of RLT lysis buffer (a highly denaturing buffer). Samples were then mixed thoroughly by pipetting or vortexing with the Vortex Mixer (Altec Lab Products, India). The cell lysate was then passed through a QIAshredder homogeniser by centrifugation at full speed for 2 minutes to shear high molecular weight genomic DNA and other cellular components which can reduce RNA binding to the silica-gel membrane. To promote adsorption of RNA to the silica-gel membrane, 70 % ethanol was then added. The lysate was transferred to the spin column. The spin column was centrifuged at 8000 x g for 15 seconds and the flow through containing denatured protein and cellular debris was discarded. A further wash with buffer RW1 removed further contaminants. To ensure no DNA contamination of RNA sample an on-column DNase digestion was performed with the RNase-Free DNase Set, according to the manufacturer´s instructions. After 15 minutes of incubation at room temperature a further wash step with 350 µl RW1 buffer was performed. A further

two wash steps with 500 µl RPE buffer containing 80% ethanol with centrifugation followed. A one minute centrifugation step at full speed was performed to ensure the membrane was dried completely. RNA was eluted in 30-50 µl of RNase-free water by centrifugation at 8000 x g for 1 minute.

2.6.1.2 RNA Bee protocol:

The protocol describes isolation of RNA with 1 ml of RNA-Bee using the following steps:

Cells were counted and lysed by adding at least 0.2 ml of RNA-Bee per 106 cells. Then 0.2 ml chloroform (Sigma-Aldrich) was added per 1 ml of RNA-Bee and shaken vigorously for 15 - 30 seconds. The samples were incubated on ice (or refrigerated at 4 ºC) for 5 minutes and the homogenates were centrifuged at 12,000 x g for 15 minutes at 4 ºC. Following centrifugation, the samples form the lower blue phenol-chloroform phase, interphase, and the upper colourless aqueous phase. RNA remains exclusively in the aqueous phase. For RNA precipitation, the aqueous phase was transferred to a clean tube, 0.5 ml of isopropanol was added, and the samples were incubated for 10 minutes at room temperature and then centrifuged at 12,000 x g for 5 minutes at 4 – 25 ºC.

Finally, the supernatant was discarded and the RNA pellet washed once with 75 % ethanol (Sigma-Aldrich), vortexing to dislodge the pellet from the side of the tube and centrifuged for 5 minutes at 7,500 x g at 4 – 25 ºC. At least 1 ml of ethanol solution was used per 1 ml of RNA-Bee used for the initial homogenisation. An additional wash with 75 % ethanol was included to improve the 260/280 ratio, which would be indicative of a greater nucleic acid purity.

At the end of the procedure, the RNA pellets were briefly air-dried for 10 minutes and the RNA was dissolved in 50 µl of RNase-free water.

2.6.2 Quantification and quality assessment of nucleic acids

RNA samples were quantified using the NanoDrop™ ND1000 (Thermo Fischer Scientific) by measuring absorbance at 260 and 280 nm (A260, and A280, respectively). Purity of RNA was determined using the A260/A280 ratio, and all

samples utilised in our experiments had a A260/A280 ratio between 1.8 and 2.1, which is the minimum to assess RNA purity as reviewed by *Udvardi et al., 2008*.

2.7 Real-Time Polymerase Chain Reaction (Real-Time PCR)

All Real-Time PCR reactions were performed on the Stratagene Mx3000p QPCR System (Qiagen, Germany) following manufacturer's recommendations and analysed with the Stratagene MxPro QPCR Software, version 4.10. All samples were run in triplicate. Triplicate no-template controls (replacing cDNA template with PCR grade water to reveal possible contamination problems) were included in each assay.

2.7.1 Reverse Transcription Polymerase Chain Reactions (RT-PCR)

The RT-PCR reactions were performed using commercially available kits and the optimum conditions were established in optimisation reactions. One available first strand cDNA synthesis kit (Quanti Tect Reverse Transcription Kit, Qiagen) was used during the study according to the manufacturers' protocol to generate cDNA for use in RT-PCR from RNA. An aliquot of 0.5 µg of total cellular RNA in RNase free water was heat denatured at 65°C for 5 minutes and then quenched on ice. A master mix was prepared on ice consisting of 2 µl of 10x Buffer RT, 2 µl of dNTP mix (5 mM each dNTP), 0.2 µl of oligo-dT primer (10 µM), 2 µl of DTT (100 mM), 1 µl of Omniscript Reverse Transcriptase and RNase free water (all Qiagen) enough to make up a 19.75 µl volume per reaction, to which 0.25 µl of rRNasin ® RNase inhibitor (Promega, USA) (40 U/µl) was added and then aliquoted into PCR tubes (Bio-Rad) containing the denatured RNA. The solution was then incubated at 37°C for 1 hour using the Bio-Rad iCycler thermal cycler. PCR reactions were set up using one commercially available PCR kit (GoTaq® PCR Core Systems, Promega). The thermal profile utilised with this kit is displayed in Table 2.1. To ensure consistency all experiments were performed on the same thermal cycler (iCycler, Bio-Rad) using the optimised conditions for the respective primers.

Step	Temperature	Time	Number of cycles
1) Initial denaturation	95ºC	2 min	1
2) Denaturation	95ºC	40 sec	30
2) Annealing	55ºC	40 sec	
2) Extension	72ºC	1 min	
3) Final extension	72ºC	5 min	1
4) Synthesis	4ºC	∞	-

Table 2.1. Summary of RT-PCR reaction cycling conditions used to generate cDNA.

The cDNA produced was used immediately for PCR or stored at -20°C.

2.7.2 Real-Time PCR primers and probes

All DNA oligonucleotides were purchased from Eurofins MWG Operon (eurofinsdna.com). As primers for the genes of interest could not be found in the existing literature, primer sets were designed to span the genes of interest.

The human and canine primers utilised for mRNA expression analysis, excluding reference genes RPL32 and HPRT, were all EMT markers.

2.7.3 Primer design

The canine and human housekeeping genes HPRT and RPL32 were designed from published sequences (NCBI Reference Sequence: NM_000194.2 and NM_001003357.1 for HPRT of human and canine origin, respectively, and NM_000994.3 and NM_001252169.1 for RPL32 of human and canine origin, respectively). All other primers were designed using the online PCR primer design tool on eurofinsdna.com. All primers are presented in Table 2.2.

Primer set		Sequence (5'-3')	Amplicon size
Human HPRT	Forward	ATGGACAGGACTGAACGTCTT	113
	Reverse	TCCAGCAGGTCAGCAAAGAA	
Canine HPRT	Forward	TTATGGACAGGACTGAGCGG	82
	Reverse	TTGAGCACACAGAGGGCTAC	
Human RPL32	Forward	AGCCATCTCCTTCTCGGCAT	105
	Reverse	ATATCGGTCTGACTGGTGCC	
Canine RPL32	Forward	CATCGATCGCTGGGCATCAT	90
	Reverse	CTGGTGCCGGATGAACTTCT	
Human E-cadherin	Forward	GGTGCTCTTCCAGGAACCTC	86
	Reverse	TAAGCGATGGCGGCATTGTA	
Canine E-cadherin	Forward	TGACAGCTACACGTTCACCG	80
	Reverse	TGCATCCTTCAAAACTCACCCT	
Human β-catenin	Forward	CAGCAGCAATTTGTGGAGGG	93
	Reverse	TCGGTTGTGAACATCCCGAG	
Canine β-catenin	Forward	GGAATGGCTACCCAAGCTGA	91
	Reverse	AAGACTGTTGCTGCCAGTGA	
Human Fibronectin	Forward	ATACCATCATCCCAGAGGTGC	101
	Reverse	GGTGGAAGAGTTTAGCGGGG	
Canine Fibronectin	Forward	GACCTAGAGGTCATCGCTGC	115
	Reverse	CAGGGCTGTTTCCTCCTGTT	
Human Vimentin	Forward	GGCTCGTCACCTTCGTGAAT	113
	Reverse	ATGCCACAGGACTCCATAC	
Canine Vimentin	Forward	GGACCAGCTCACCAACGACA	99
	Reverse	AGCATCTCCTCTTGCAACTTCTC	

Table 2.2. Primer sequences for human and canine genes of interest.

2.7.4 Real-Time PCR reactions

The Real-Time PCR reactions were performed using Platinum® SYBR Green qPCR SuperMix-UDG (Invitrogen) master mixes with a reaction volume of 25 µl in a 96 well plate format. Real-Time PCR reactions contained 12.5 µl of SYBR Green mix, 1.25 µl of each primer (forward and reverse), 0.5 µl of a one in ten dilution of reference dye (ROX) and 9.5 µl of a one in twenty dilution of cDNA synthesis reaction as template. The cycling conditions used are summarised in Table 2.3.

Step	Temperature	Time	Number of cycles
1) UDG Incubation	50ºC	2 min	1
2) Taq activation	95ºC	2 min	1
3) Denaturation	95ºC	15 sec	30
3) Annealing and extension	55-60ºC	30 sec	
4) Dissociation curve	95ºC	1 min	1
	60ºC	30 sec	
	95ºC	15 sec	
	25ºC	30 sec	

Table 2.3. Thermal profile for qRT-PCR.

2.7.5 Data Analysis of Real-Time results

All analyses of relative gene expression levels were performed using the comparative delta-delta CT method (Pfaffl, 2001). All data analyses and graphs were generated using Microsoft Office Excel 2003.

2.7.6 Primer efficiency

To determine primer efficiency cDNA from untreated and TGF-β-stimulated MCF7 and REM cell lines were used as template. Serial dilutions of cDNA reaction mixtures from 1:30 to 1:960 were made and Real-Time PCR reactions were performed in three technical replicates with each primer set individually.

The cycling threshold (Ct) or Crossing point (Cp) values of each of the dilutions were plotted against the cDNA concentrations and the slopes were calculated from the graphs. High primer efficiency produces graphs with a high correlation coefficient (Pearson's correlation coefficient r>0.99) and with high linearity in the investigative range. One hundred per cent efficiency corresponds to a perfect doubling of template at every cycle, but the acceptable range is 90-110% for assay validation as reviewed by *Taylor et al., 2011*. This efficiency range corresponds to standard curve slopes of -3.6 to -3.9.

2.7.7 Relative Expression Analysis

Following primer efficiency estimations, Real-Time PCR reactions were performed in triplicate for each sample. To determine the fold change in treated samples compared to control samples, the Ct values (also known as delta Ct) of the target genes and reference genes were obtained by subtracting the treated sample values from the control sample values. This was so that increases will have a positive result and decreases a negative result for the delta Ct value (ΔCt). The efficiency (E) of each primer was also taken into account to calculate the fold increase in each assay by submitting the efficiency percentage to the n^{th} power as shown in Figure 2.1. After calculating the fold increase with both target genes and reference genes, we divided the fold change in the target genes by the fold change in the reference genes in order to normalise and correct the data for each assay as expressed in a universal ratio formula described by *Pfaffl, 2001* (Pfaffl, 2001) as shown in Figure 2.2.

Fold change in target or reference gene:

$$\text{Fold change} = (E)^{n}$$

$$\text{Fold change} = (\text{primer efficiency})^{\text{control sample value – treated sample value}}$$

Figure 2.1. Fold change formula for target or reference genes. E= efficiency; n= ΔCt (treated sample values – control sample values).

It is expected that the reference genes have very close Ct values and the ratio in the treated and control samples would be close to 1. This would suggest that they are good reference genes for the experiment.

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control\text{-}treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control\text{-}treated)}}$$

Figure 2.2. Normalisation of fold change for each assay. E_{target}= primer efficiency of the target gene; E_{ref}= primer efficiency of the reference gene; ΔCt target= ΔCt of the target gene; ΔCt ref= ΔCt of the reference gene.

2.8 *In vitro* scratch assay

TGF-β-stimulated and unstimulated cells were passaged and counted as previously described in Chapter 2.2, after TGF-β stimulation. REM and CMC cells were stimulated with 10 ng/ml of TGF-β every 24 hours for 6 days, changing media and passaging when necessary. After 6 days of stimulation, cells were seeded at high density (0.8-1.0 X 106 cells) into 6 well plates and allowed to reach confluency (usually after 24 hours). A scratch was then created with a 200 µl pipette tip in the monolayer of each well. Following the creation of the scratch, the media was removed and the wells were washed with 2 ml PBS per well until all floating cells were removed and a clean, sharp edge of cells could be visualised in all wells by phase contrast light microscopy. The cells were photographed using the 10x

objective at different time points. The area photographed was marked by permanent marker on the lid and the bottom outer part of each well so that the same areas could be photographed at every time point.

The gap width was then measured at ten different places within each scratch´s length. The mean gap width was then determined for each time point and compared to the starting mean gap width so that the closing percentage could be determined.

The mean gap width was then expressed as relative migration distance covered by cells closing the wound (scratch). Then, the percentage closing was compared between TGF-β-stimulated cells and the untreated group. In order to calculate these measurements, the following formula was used.

Formula: Relative migration distance (%) = 100 (A-B) / A.

A= width of cell wound before incubation, and B= width of cell wound after incubation.

2.9 Magnetic cell sorting (MACS sorting)

The separation columns, magnetic separator and the CD133 MicroBead Kit were all purchased from Miltenyi Biotec Inc., Germany. Cells were harvested and counted as described in Chapter 2.2. A maximum of 1x108 cells per sample were pelleted by centrifugation at 100 x g for 5 minutes and resuspended in 300 µl of chilled separation buffer. One hundred µl of FcR blocking reagent and 100 µl of CD133 MicroBeads were added and the solution was incubated for 30 minutes at 4°C with rotation.

The cells were washed by adding 5 ml of cold separation buffer and centrifuged at 300 x g for 10 minutes at 4°C before resuspension in 1 ml of separation buffer and transfer to a pre-wet separation column mounted on the magnetic separator. The column was washed three times with 3 ml of separation buffer and the flow through containing the negative fraction was collected. The

column was then removed from the magnetic separator and the labelled fraction was flushed out in 5 ml of separation buffer using the plunger. The labelled and unlabelled fractions were counted and seeded at required density for chemosensitivity assays.

2.10 Chemosensitivity assay

The CellTiter-Glo® Luminescent Cell Viability assay (Promega, USA) determines the relative number of metabolically active cells present in culture based on quantification of the ATP present. The luminescence signal generated is proportional to the amount ATP present which again is directly proportional to the number of metabolically active cells present in culture.

CD133+ and CD133- cells (with and without TGF-β stimulation) were counted and resuspended in DMEM media at 1 x 104 cells / ml and 50 µl of cell suspension was seeded into each well of an opaque-walled 96-well plate (Sigma-Aldrich), therefore a total number of 500 cells / well. The assay was run in triplicate for each group of cells and for each chemotherapeutic´s concentration (10 concentrations for each drug). Controls included no cell and no drug treatments. The plates were incubated overnight at 37ºC, 5% CO2.

The drugs used were doxorubicin (Pharmacia/Pfizer, UK) and mitoxantrone hydrochloride (Baxter Healthcare Ltd, UK) and were prepared at a stock concentration of 200 µM in DMEM. A serial dilution of each drug in DMEM was prepared in triplicate for each cell line and controls, and 50 µl of each drug dilution was added per well and incubated for 72 hours at 37ºC, 5% CO2.

To generate the ATP standard curve, 50 µl of a 10mM ATP solution (Life Technologies) were added to 4.95 ml of culture medium in order to produce a 100 µM concentration. For each plate, 4 Eppendorf tubes were prepared in which medium was used to further serially dilute 1:10 the 100 µM ATP solution in order to make 10 µM, 1 µM, 100 nM and 10 nM dilutions, of which 50 µl were added to wells of each plate just before analysis.

The CellTiter-Glo substrate was mixed with the CellTiter-Glo buffer to produce CellTiter-Glo reagent. Reagents and culture plates were equilibrated to room temperature before assaying. One hundred µl was added to each well of an opaque-walled 96-well plate (Sigma-Aldrich). Plates were incubated on an orbital shaker for 2 minutes and then immediately incubated at room temperature for 10 minutes to stabilise the luminescent signal. Luminescence was recorded using the Perkin Elmer 1420 Multilabel Counter Victor3™ plate reader (Perkin Elmer).

Triplicate vehicle-only (no chemotherapeutic drug) controls were used in each plate. Luminescence reading from these wells were used for normalisation of the chemotherapy-exposed cell luminescence data to represent 100% cell viability. All other readings from chemotherapy-exposed cells were compared to the normalised controls in order to give accurate percentage of cell viability.

2.11 microRNA expression analysis

RNA was extracted from REM as described in 2.6.1.2, then sent to Edinburgh Genomics-Roslin Institute (Centre for Comparative and Functional Genomics, The Roslin Institute, The University of Edinburgh, UK) for microRNA sequencing. The RNA extracts were chosen from a TGF-β time course experiment during which harvested protein samples from cell homogenates showed changes in protein expression suggesting that they underwent EMT. The analysed samples were:

REM cells without stimulation (control group)

REM cells treated with vehicle carrier after days 1, 10, 17 and 23 (REM mock)

REM cells treated with 10 ng/ml TGF-β after days 1, 10, 17 and 23 (REM TGF-β)

REM cells at day 23 with TGF-β withdrawal after 19 days (REM wd-19)

The miRNA library preparation was performed according to the preparation guide provided by Illumina® Truseq™ Small RNA (Illumina, Inc., USA), using total RNA as starting material. Both 5' and 3' RNA adapters were added to 1 µg total RNA. These adapters were specially designed so that they would preferentially ligate small RNAs. Reverse transcription PCR was then performed to amplify the adapter-

ligated small RNAs. The products were selected according to their size utilising polyacrylamide gel electrophoresis (PAGE), thereafter molecules of 145-160 bp were excised from polyacrylamide gel and subsequently purified and concentrated through ethanol precipitation. Validation of the miRNA libraries was performed with a Bioanalyzer 2100 (Agilent Technologies, Inc., USA). A pool containing all libraries was sequenced at Edinburgh-Genomics.

The reads from the microRNA sequencing experiment were mapped to known canine microRNAs using Novoalign™ version 3.0 (Novocraft Technologies Sdn Bhd, Malaysia). The unmapped reads were then mapped to human microRNAs. A spreadsheet of matched reads was constructed showing rows as microRNAs and columns as experiments. These were all normalised to reads per million (RPM) mapped sequences.

For the differential expression analysis, empirical analysis of digital gene expression data in R (EdgeR version 3.10.2, Bioconductor, USA) was utilised to compare the mock-treated and TGF-β-stimulated samples at each time point. We also compared REM TGF-β withdrawal samples with REM mock-treated and REM TGF-β-stimulated samples after day 23. No biological replicates were used in this experiment.

Normalised counts were calculated as number of reads / total number of reads mapped (millions).

Counts were analysed for:

- Normalised counts for each microRNA
- logFC: log(2) fold change. Positive values would be upregulated and negative values downregulated.
- logCPM: the average log(2) CPM (counts per million). High values would show that microRNA is highly expressed and low values would show lowly expressed microRNA.
- P-value: unadjusted p-values for each comparison.

- FDR (False discovery rate): adjusted p-value using the Benjamini and Hochberg correction (Benjamini and Hochberg, 1995).

Lowly expressed microRNAs were filtered out. Due to the experimental design, there were not many microRNAs that passed the FDR≤ 0.05 filter. This would be expected when there are no biological replicates.

2.12 MicroRNA validation

qRT-PCR was performed using the same RNA that was utilised for miRNA expression analysis. qRT-PCR was performed as in Chapter 2.7 and was followed with specific microRNA expression assays targeting mature miRNA sequences from TaqMan®, Life Technologies™. These assays use a target-specific stem-loop reverse transcription primer for each microRNA targeted as displayed in Table 2.4. This method was utilised as miRNAs are constructed of only 17-24 nucleotides but regular qRT-PCR methods typically use templates of at least double the length of each primer. This means that the minimum length of each target would typically be a minimum of 40 nucleotides. As miRNAs are too short for such methodologies, the method used herein utilises a specific reverse transcription primer containing a stem-loop structure which transcribes a longer miRNA/stem-loop cDNA target, which is then sequenced using primers designed for the miRNA/stem-loop cDNA target (Kramer, 2011).

Specific mature microRNA sequences utilised:

Cfa-miR-380	UAUGUAAUAUGGUCCACGUCU
Cfa-miR-381	UAUACAAGGGCAAGCUCUCUGU
Cfa-miR-410	AAUAUAACACAGAUGGCCUGU
Cfa-miR-411	AUAGUAGACCGUAUAGCGUACG

Table 2.4. Mature microRNA sequences targeted with their specific stem-loop reverse transcription primer.

2.13 Statistical analysis

Statistical analyses of data were performed using Minitab© 15 Statistical Software (Minitab Ltd., UK). P-values <0.05 were considered statistically significant. Non-parametric testing was performed. Mann Whitney U-tests were used to compare differences between two samples. Adjusted p-values for miRNA sequencing were based on the Benjamini and Hochberg correction.

Chapter 3: Induction of EMT confers a mesenchymal-like phenotype and migratory properties to breast cancer cells

3.1 Abstract

Breast cancer is a very common disease in humans and dogs. It is the most common cancer in both women and un-spayed female dogs. The metastatic potential of mammary carcinoma is very high, in affected patients. Metastatic cells can migrate, invade and colonise different organs. These secondary tumours are often more aggressive than the primary tumour, and may be lethal to the patient. Epithelial to Mesenchymal Transition (EMT) has been implicated as a driver of metastasis. During EMT, epithelial cells acquire a mesenchymal-like phenotype, which enables them to move easier through surrounding tissues and vessels to migrate and invade different organs.

The aims of this study were first to establish the level of expression of epithelial and mesenchymal markers in breast cancer cells of dogs, cats and humans after confirming morphological changes related to a mesenchymal-like phenotype (spindle shape, separation from groups of cells and front to back polarity). Mammary carcinoma cells from all three species were stimulated with TGF-β to induce EMT and each cell line tested showed characteristic mesenchymal changes. After TGF-β stimulation cells acquired migratory characteristics, observed by *in vitro* migration and invasion assays. These changes confirm the acquisition of a mesenchymal-like phenotype with migratory and invasive characteristics in breast cancer cells through a TGF-β-induced EMT process.

3.2 Introduction

Epithelial to Mesenchymal Transition (EMT) is a process involved in embryogenesis, carcinogenesis, and metastasis as described in Chapter 1.5. The Transforming Growth Factor-Beta (TGF-β) pathway and its associated transcription factors are crucial for EMT induction, during which epithelial cells lose their defining characteristics and acquire mesenchymal properties (Voulgari and Pintzas, 2009, reviewed by Huber et al., 2005 and Moustakas and Heldin, 2007) as previously described in Chapter 1.10. Furthermore, during EMT epithelial cells undergo cytoskeletal changes, acquiring increased motility and invasiveness (reviewed by Savagner et al., 1994, and Thiery and Chopin, 1999). Interestingly, in some cases cells progress through a partial EMT, expressing both, mesenchymal and epithelial markers during the process (reviewed by Yang and Weinberg, 2008), indicating that phenotypical changes may not be entirely specific for a mesenchymal or an epithelial cell even though they have the ability to migrate and invade.

EMT can facilitate cell migration towards different tissues in the organism, enabling metastatic cells to invade other organs due to a type III EMT as reviewed by *Kalluri and Weinberg, 2009*. Once these cells reach a new organ or niche, they can produce a secondary, or metastatic tumour and become more harmful (reviewed by Kalluri and Weinberg, 2009).

EMT has been studied at different levels in different species, especially humans and mice, as previously described in Chapters 1.5 and 1.12, but to date there are few significant studies regarding type III EMT in companion animals including dogs and cats, including studies carried out by *Salgado et al. in 2014 and Fonseca-Alves et al. in 2015*, mentioned in Chapter 1.16 (Salgado et al., 2014 and Fonseca-Alves et al., 2015).

An important difference between humans and companion animals regarding metastasis is the predilection for distant metastatic sites during breast cancer progression. In humans, bone and lungs are the first invaded organs in that respective order (Chen et al., 2010, Coleman, 2006), whereas in dogs and cats, the lungs tend to be the first organs affected by distant breast cancer metastasis, as reviewed by *Nelson*

and Couto, 2000, Ogilvie, 2006, Lana and Withrow, 2007, and Sorenmo et al., 2013; and the pathophysiology of cancer, diagnostic and therapeutic approaches vary between these species (reviewed by Sorenmo, 2003, , Sorenmo et al., 2011, and Sorenmo et al., 2013). The diagnostic approach in humans differs from that in companion animals as humans often present at an earlier stage following routine or self-examination, and some diagnostic tools are not affordable or available in animals, especially in developing countries. However, if we consider the fact that dogs and cats are more similar to humans than are conventional laboratory animals (reviewed by MacEwen, 1990, Vail and MacEwen, 2000, Printz, 2011, Liu et al., 2014), the current studies were designed to specifically investigate TGF-β-induced EMT in human, canine, and feline mammary carcinoma cell lines, thus investigating whether dogs and cats are useful animal models for the study of EMT in cancer progression.

Induction of EMT is directly proportional to the repression of E-cadherin as described in Chapter 1.5, in which different downstream effectors in the TGF-β pathway play important roles. E-cadherin expression is inversely proportional to the tumour grade and stage, and patient prognosis as reviewed by *Hirohashi, 1998, Thiery, 2002, Peinado et al., 2004, and Schmalhofer et al., 2009*.

A subunit of the cadherin protein complex, β-catenin, acts by binding E-cadherin and α-catenin to the actin cytoskeleton and is essential for keeping cells attached to each other (reviewed by Schmalhofer et al., 2009, Acloque et al., 2008, Brabletz et al., 2005) as shown in Figure 1.2. The upregulation of mesenchymal promoters like vimentin and fibronectin is enabled by repression of cadherins including E-cadherin and β-catenin. Vimentin enables cells to change their shapes giving strength and flexibility to their cytoskeletons in order to prevent damage during migration through surrounding tissues as reviewed by *Goldman et al., 1996*. This protein is an intermediate filament polypeptide present in the cytoskeleton of mesenchyme-derived cells but not in epithelia-derived cells which has been shown to play a role in type I and type II EMT (reviewed by Zeisberg and Neilson, 2009, Dellagi et al., 1983, Franke et al., 1978). Furthermore, fibronectin is an extracellular matrix glycoprotein which is essential for vertebrate development and it plays an

important role in cell adhesion, migration, cytoskeletal organisation, apoptosis and tissue remodelling (Williams et al., 2008). It is continuously produced by adherent cells (Darribere and Schwarzbauer, 2000). This glycoprotein plays an important role in guiding cells so that they can reach their new niche where it binds to collagen and fibrin to enable cells to colonise the new microenvironment (Williams et al., 2008, Darribere and Schwarzbauer, 2000, Hao et al., 2004).

Upregulation and downregulation of transcription factors such as Twist and ZEB1 can potentially lead to a series of molecular changes in the epithelial and mesenchymal proteins mentioned above, as demonstrated by *Comijn et al., 2001, Yang et al., 2004, and Eger et al., 2005*, and reviewed by *Peinado et al., 2007*. Twist and ZEB1 are known to trigger EMT by downregulating E-cadherin expression (Bae, 2013). Thus the downregulation of E-cadherin is considered the hallmark of EMT (Bolos et al., 2003).

In this study we intended to stimulate canine and feline mammary carcinoma cell lines with TGF-β and to observe if they underwent an EMT, as has been shown in human mammary cancer cell models (Bakin et al., 2000, Dalal et al., 1993, Xie et al., 2004). A human mammary carcinoma cell line, MCF7, was utilised as a control, and in order to assess the similarities and differences between human, feline and canine mammary carcinoma cells undergoing TGF-β-induced EMT. The aims of this study were to clarify the role of EMT as a relevant transition process in cancer research in animals to promote a better understanding of the disease and pursue better diagnostics and therapeutics in dogs and cats.

3.3 Results

3.3.1 TGF-β-induced EMT confers cells a mesenchymal morphology

Cells with epithelial characteristics have a round shape and typically are found in groups due to their tight junctions. They have a top to bottom polarity facilitating their attachment to their basement membrane and each other and their functional roles. These cells are polarised such that the bottom is defined as basal, and the top as apical. In contrast, mesenchymal cells have a more elongated shape and their polarity is switched to a front to back polarity. Moreover, their intercellular junctions are inadequate to stay attached to their basement membrane and each other, therefore they do not form large groups, and easily migrate (reviewed by Acloque et al., 2009). Canine mammary carcinoma (REM), feline mammary carcinoma (CMC) and human mammary carcinoma (MCF7) cell lines were stimulated with 10 ng/ml of TGF-β every 24 hours for 6 days. TGF-β-stimulated cells showed morphological changes consistent with a more mesenchymal phenotype after 6 days of treatment compared to vehicle-treated cells. Cells of all species became more elongated and separated away from each other indicating that they were losing their tight junctions and changing their polarity from a top to bottom to a front to back polarity. We observed that CMC cell line showed individual cells with a more elongated or spindle shape separating from other cells at day 6 of TGF-β stimulation, indicating loss of tight junctions and remodelling of cytoskeleton. Interestingly, vehicle-treated cells also showed spindle shapes by day 6 but in contrast to TGF-β-stimulated cells, they remained closely attached to each other as shown in Figure 3.1. REM cells also showed a more mesenchymal morphology after stimulation with TGF-β. A spindle shape and a greater separation of cell groups was evident at day 6 of treatment in REM cells compared with the vehicle-treated group as shown in Figure 3.2. Furthermore, the human breast cancer cell line, MCF7 also showed morphological changes consistent with a more mesenchymal phenotype after TGF-β exposure. We observed that MCF7 cells did not show a very pronounced spindle shape, but they did display cytoplasmic widening and detachment from each other at day 6 of treatment as shown in Figure 3.3.

Figure 3.1. Morphological changes in feline mammary carcinoma (CMC) cell line after TGF-β stimulation. 0 ng/ml TGF-β = vehicle-treated cells. 10 ng/ml TGF-β = cells stimulated with 10 ng/ml of TGF-β. Images were taken at days 0, 1, 2, 3 and 6 of stimulation with TGF-β or vehicle. Scale bar represents 50 µm.

Figure 3.2. Morphological changes in canine mammary carcinoma (REM) cell line after TGF-β stimulation. 0 ng/ml TGF-β = vehicle treated cells. 10 ng/ml TGF-β = cells stimulated with 10 ng/ml of TGF-β. Images were taken at days 0, 1, 2, 3 and 6 of stimulation with TGF-β or vehicle. Scale bar represents 50 µm.

Figure 3.3. Morphological changes in human mammary carcinoma (MCF7) cell line after TGF-β stimulation. 0 ng/ml TGF-β = vehicle treated cells. 10 ng/ml TGF-β = cells stimulated with 10 ng/ml of TGF-β. Images were taken at days 0, 1, 2, 3 and 6 of stimulation with TGF-β or vehicle. Scale bar represents 50 µm.

Figure 3.4 is a composite of photomicrographs showing the differences between the canine, feline and human mammary carcinoma cells studied before and after undergoing TGF-β-induced EMT, with evident mesenchymal traits at day 6 of treatment with 10 ng/ml TGF-β. All cell lines showed individual cells separating from the main group of cells.

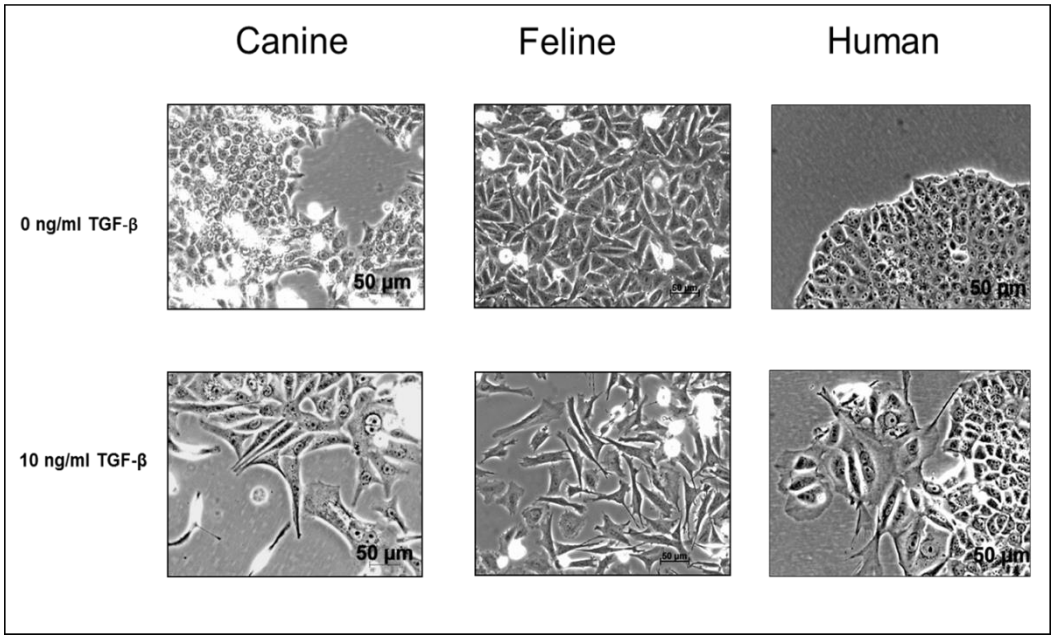

Figure 3.4. Comparison between the three different cell lines, showing that canine (REM), feline (CMC) and human (MCF7) mammary carcinoma cell lines display evident mesenchymal features at day 6 of TGF-β stimulation compared to vehicle-treated cells. 0 ng/ml TGF-β = vehicle-treated cells. 10 ng/ml TGF-β = cells treated with 10 ng/ml of TGF-β. Scale bar represents 50 µm.

3.3.2 TGF-β leads to EMT by downregulation of epithelial and upregulation of mesenchymal proteins in breast cancer cells

In order to further assess whether epithelial breast cancer cells in dogs, cats and humans acquired mesenchymal traits after TGF-β stimulation, we analysed the expression of proteins that are often changed during EMT in the three cell lines studied (REM, CMC and MCF7). Cells were treated with 10 ng/ml of TGF-β every 24 hours, and harvested after 6 days. The protein expression of epithelial markers E-

cadherin and β-catenin, and mesenchymal markers vimentin and fibronectin, were analysed by western blot. We found that epithelial markers E-cadherin and β-catenin are downregulated and mesenchymal markers vimentin and fibronectin are upregulated after TGF-β treatment as shown in Figure 3.5. These results were consistent across the species. The transcriptional repression of proteins, such as cadherins (E-cadherin and β-catenin), characteristic of EMT, can be accomplished by a range of transcription factors including ZEB1 and Twist as reviewed by *Savagner et al., 1994, and Thiery and Chopin, 1999*.We found that Twist was upregulated in REM cells, and ZEB1 was upregulated in CMC cells compared to untreated cells. These findings confirm that TGF-β is a promoter of EMT by repressing E-cadherin activity and enhancing upregulation of mesenchymal gene expression. This is consistent in dog, cat and human mammary carcinoma cells.

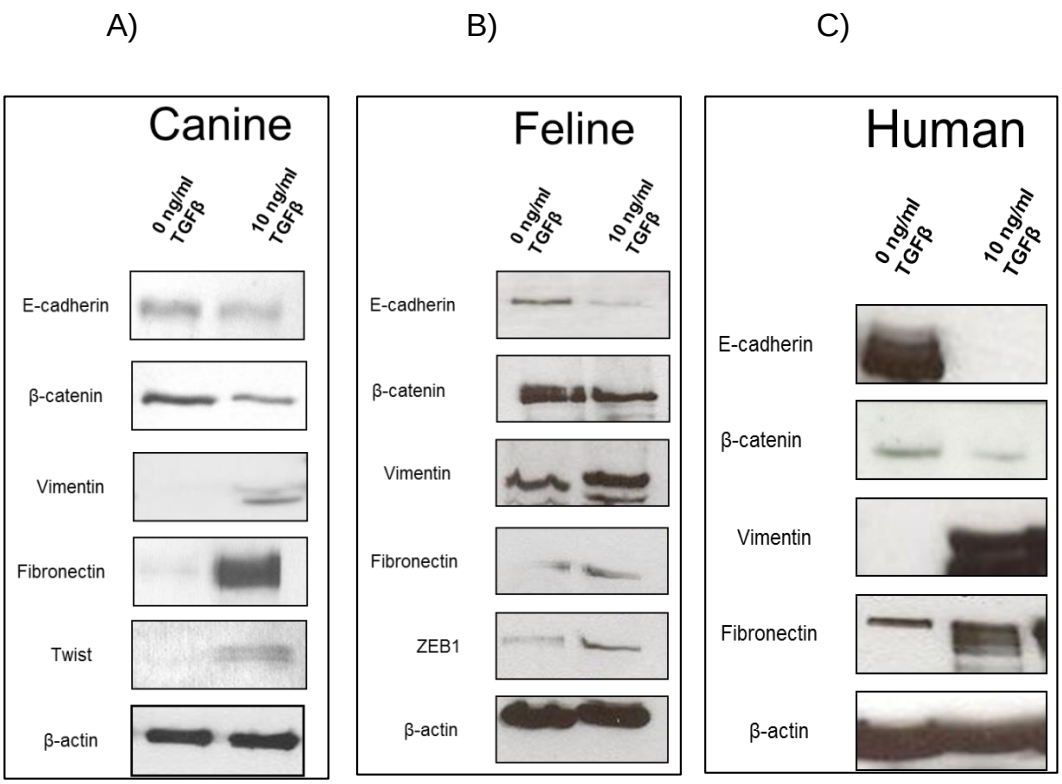

Figure 3.5. EMT-associated changes at the protein expression level after treatment with 10 ng/ml of TGF-β compared with untreated cells. A) Canine mammary carcinoma cells (REM) show down-regulation of epithelial markers (E-cadherin and β-catenin) and up-regulation of mesenchymal markers (vimentin and fibronectin) and transcription factor Twist. B) Feline mammary carcinoma cells (CMC) show down-regulation of epithelial markers and up-regulation of mesenchymal markers and transcription factor ZEB1. C) Human mammary carcinoma cells (MCF7) show down-regulation of epithelial markers and upregulation of mesenchymal markers. B-actin was used as a loading control for all cell samples. All proteins were expressed at the expected molecular weights. E-cadherin = 120 kDa, β-catenin = 92 kDa, vimentin = 57 kDa, fibronectin = 240 kDa, Twist = 28 kDa, ZEB1 = 124 kDa and β-actin = 45 kDa. Thirty µg of protein was loaded for each immunoblot.

3.3.3 EMT changes expressed at the transcriptional level in canine and human cell lines

The mRNA expression levels of EMT markers were determined by qRT-PCR after cells were treated with 2.5, 5 and 10 ng/ml of TGF-β for 4 and 8 days, and compared with untreated cells for 8 days. Morphological changes were seen by day 6 of treatment with TGF-β, as previously described in Chapter 3.3.1. We decided to assess the expression of epithelial and mesenchymal markers at the transcriptional level at day 4 and day 8 to investigate whether the morphological changes were supported by a mRNA expression changes supportive of EMT. We observed that

cells did not show downregulation of epithelial characteristics' mRNA by day 4 of treatment (data not shown). Whereas, by day 8 cells show significant changes in their mRNA expression of epithelial and mesenchymal markers, confirming that they maintain a mesenchymal phenotype at the transcriptional level at least 48 hours after the acquisition of morphological changes.

We observed that TGF-β-stimulated MCF7 and REM cells showed anticipated transcriptional changes given the EMT associated morphological and protein changes. Epithelial marker RNA expression was downregulated and mesenchymal marker RNA expression was upregulated at different time points with different concentrations of TGF-β after 8 days. All calculations for the relative expression analysis were made according to the Pfaffl method described in Chapter 2.7.7. The reference genes utilised were canine and human RPL32 for REM and MCF7 cell lines, respectively. All these experiments were repeated three times.

The RNA expression of the epithelial markers E-cadherin and β-catenin was downregulated in TGF-β-stimulated MCF7 cells in comparison to the untreated cells at day 8 with different concentrations of TGF-β. This difference was proved significant, as depicted in Figures 3.6 and 3.7, respectively. Moreover, RNA expression of the mesenchymal marker vimentin was upregulated at day 8 as depicted in Figure 3.8.

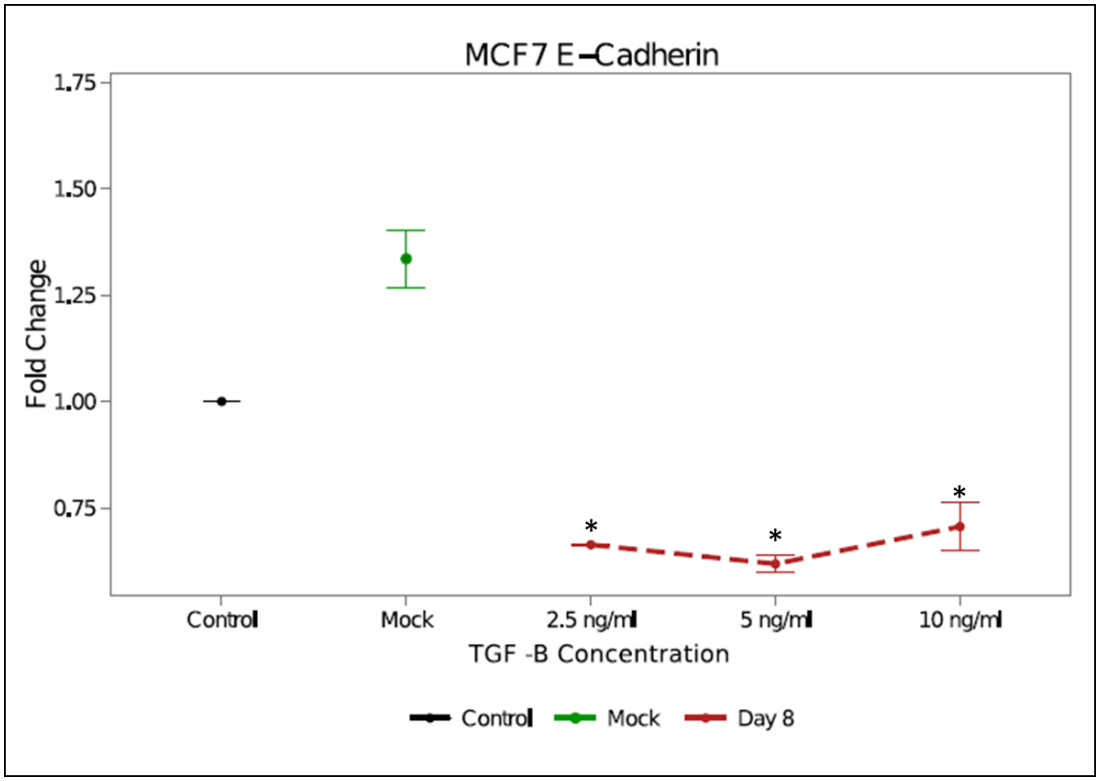

Figure 3.6. E-cadherin is downregulated in MCF7 cells after 8 days of TGF-β stimulation at 2.5, 5 and 10 ng/ml. Control = untreated cells, Mock = vehicle-treated cells for 8 days with the equivalent of 10 ng/ml of TGF-β, 2.5 ng/ml = cells stimulated with 2.5 ng/ml of TGF-β, 5 ng/ml = cells stimulated with 5 ng/ml of TGF-β, 10 ng/ml = cells stimulated with 10 ng/ml of TGF-β. Day 8 = cells harvested at day 8 of treatment. We found statistically significant differences in E-cadherin expression between cells harvested after 8 days of treatment with TGF-β and control and vehicle-treated control cells. P values comparing TGF-β- stimulated samples with control cells: * = p value ≤ 0.05.

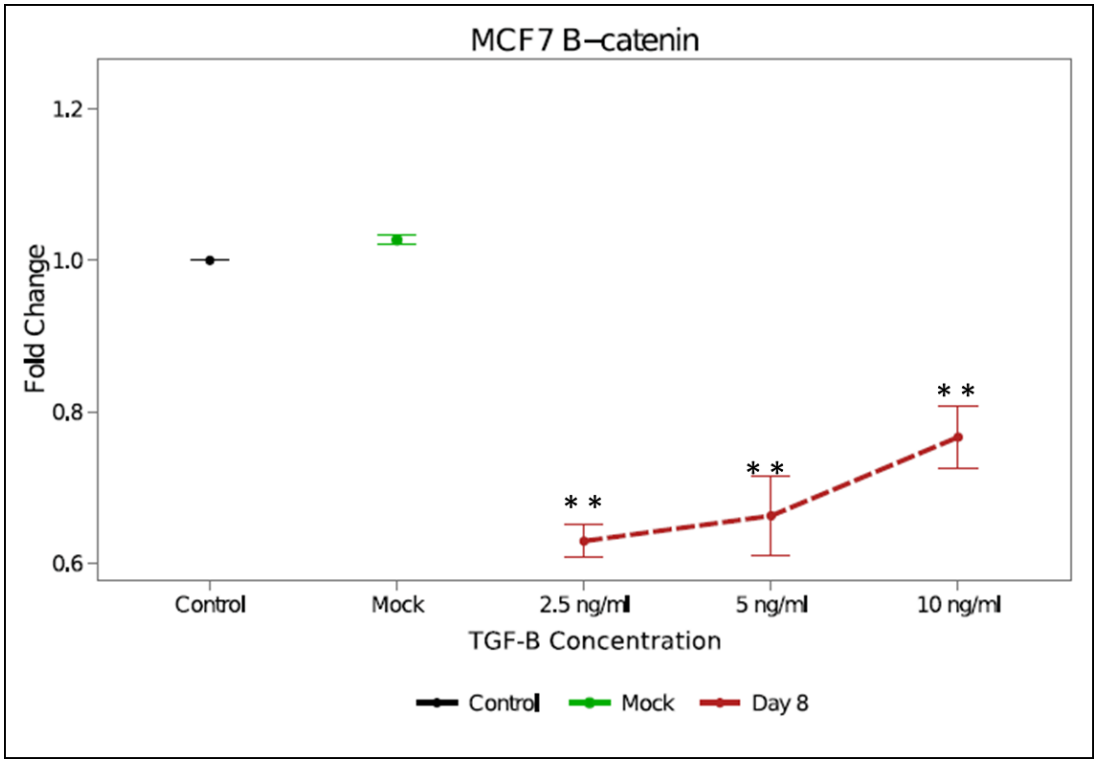

Figure 3.7 β-catenin is downregulated in MCF7 cells after 8 days of TGF-β stimulation at 2.5, 5 and 10 ng/ml. Control = untreated cells, Mock = vehicle-treated cells for 8 days with the equivalent of 10 ng/ml of TGF-β, 2.5 ng/ml = cells stimulated with 2.5 ng/ml of TGF-β, 5 ng/ml = cells stimulated with 5 ng/ml of TGF-β, 10 ng/ml = cells stimulated with 10 ng/ml of TGF-β. Day 8 = cells harvested at day 8 of treatment. We found statistically significant differences in β-catenin expression between cells harvested after 8 days of treatment with TGF-β and control and vehicle-treated control cells. P values comparing TGF-β-stimulated samples with control cells: ** = p value ≤ 0.005.

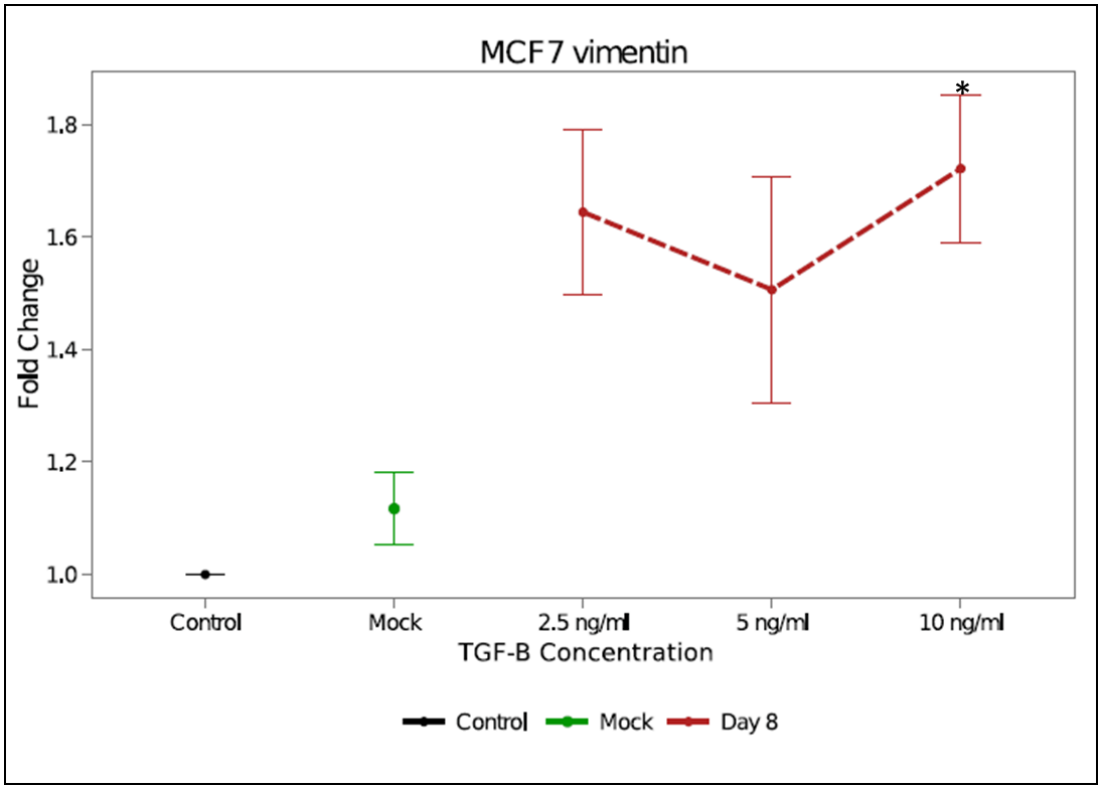

Figure 3.8. Upregulation of vimentin following exposure to TGF-β (2.5, 5 and 10 ng/ml) at day 8 of stimulation. Control = untreated cells, Mock = vehicle-treated cells for 8 days with the equivalent to 10 ng/ml of TGF-β, 2.5 ng/ml = cells stimulated with 2.5 ng/ml of TGF-β, 5 ng/ml = cells stimulated with 5 ng/ml of TGF-β, 10 ng/ml = cells stimulated with 10 ng/ml of TGF-β. Day 8 = cells harvested at day 8 of treatment. We found statistically significant differences in vimentin expression between cells harvested after 8 days of treatment with TGF-β and control and vehicle-treated control cells. P values comparing TGF-β-stimulated samples with control cells:* = p value ≤ 0.05.

REM cells did not exhibit an anticipated significant change of epithelial marker mRNA expression after TGF-β stimulation, shown in Figure 3.9 and Figure 3.10. We would expect epithelial markers to be downregulated after TGF-β treatment; however, they were highly upregulated. Nevertheless, these samples showed an upregulation of the mesenchymal marker fibronectin after 8 days of treatment at all concentrations of TGF-β compared with the untreated cells at day 8, as shown in Figure 3.11. These results show that at the mRNA level REM cells do not lose their epithelial characteristics but do acquire mesenchymal properties as discussed below.

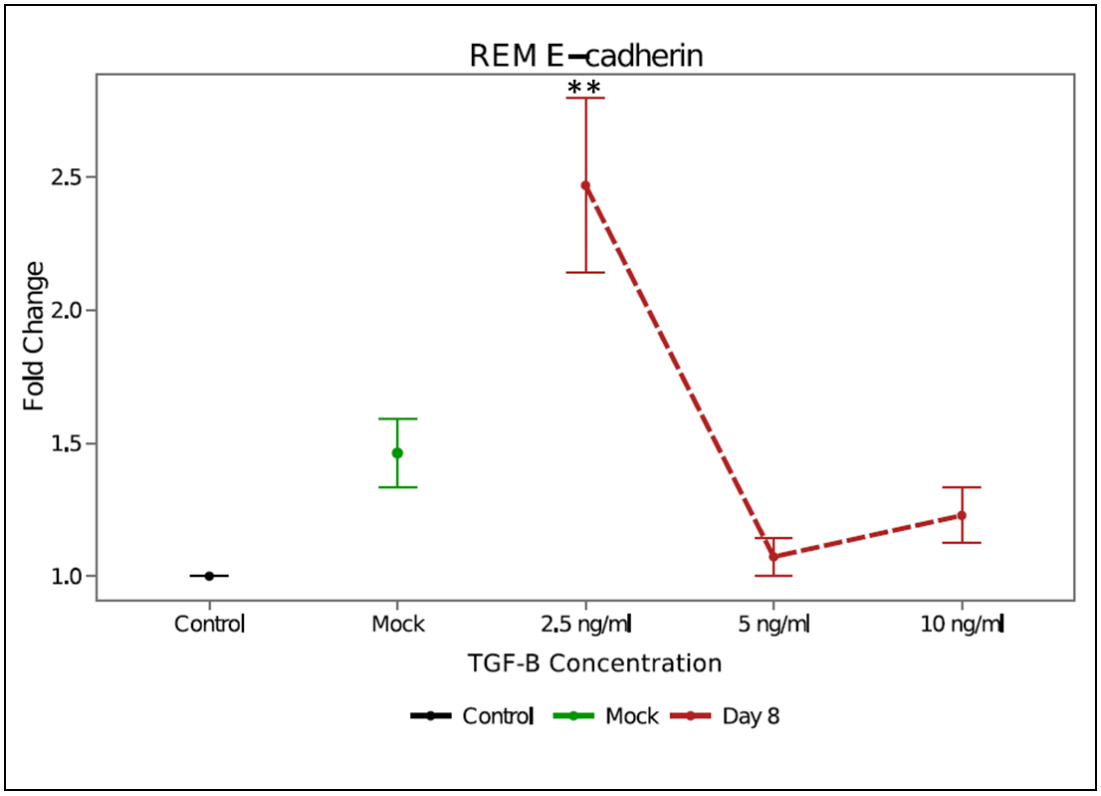

Figure 3.9. REM cells do not show downregulation of E-cadherin after TGF-β stimulation at day 8. Control = untreated cells, Mock = vehicle-treated cells for 8 days with the equivalent of 10 ng/ml of TGF-β, 2.5 ng/ml = cells stimulated with 2.5 ng/ml of TGF-β, 5 ng/ml = cells stimulated with 5 ng/ml of TGF-β, 10 ng/ml = cells stimulated with 10 ng/ml of TGF-β. Day 8 = cells harvested at day 8 of treatment. P values comparing TGF-β-stimulated samples with control cells: ** = p value ≤ 0.005.

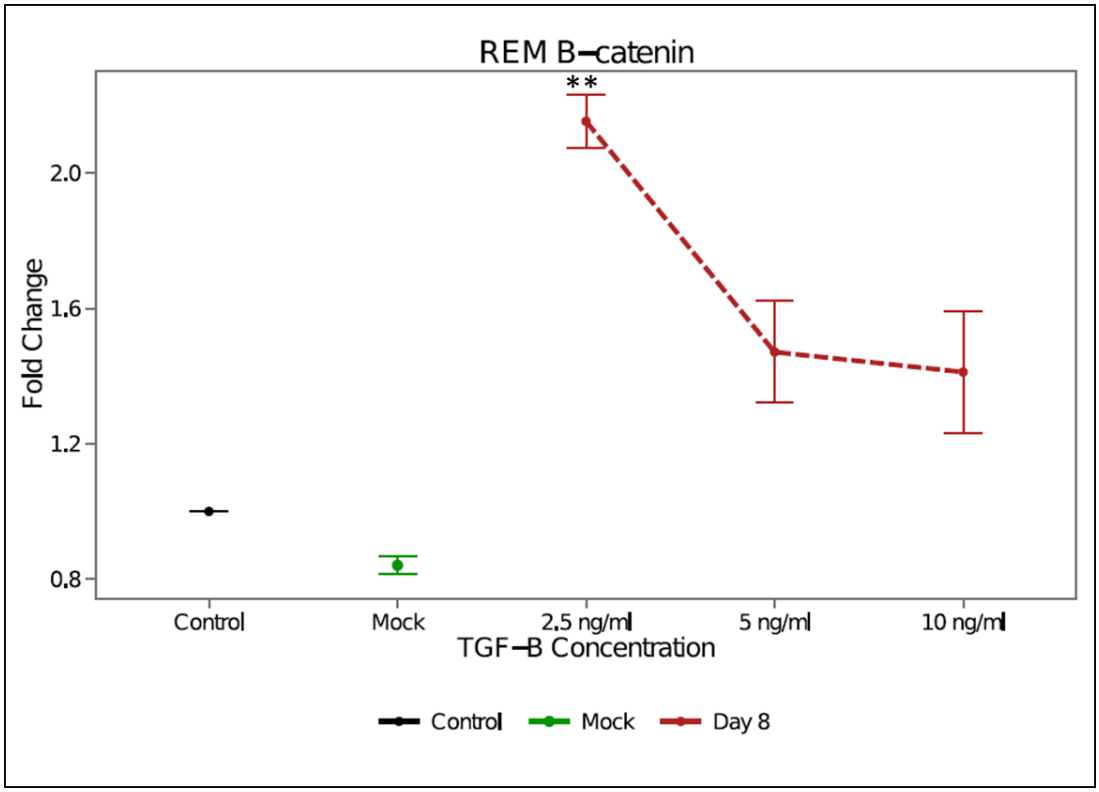

Figure 3.10. REM cells do not show downregulation of β-catenin after TGF-β stimulation at day 8. Control = untreated cells, Mock = vehicle-treated cells for 8 days with the equivalent of 10 ng/ml of TGF-β, 2.5 ng/ml = cells stimulated with 2.5 ng/ml of TGF-β, 5 ng/ml = cells stimulated with 5 ng/ml of TGF-β, 10 ng/ml = cells stimulated with 10 ng/ml of TGF-β. Day 8 = cells harvested at day 8 of treatment. P values comparing TGF-β-stimulated samples with control cells: ** = p value ≤ 0.005.

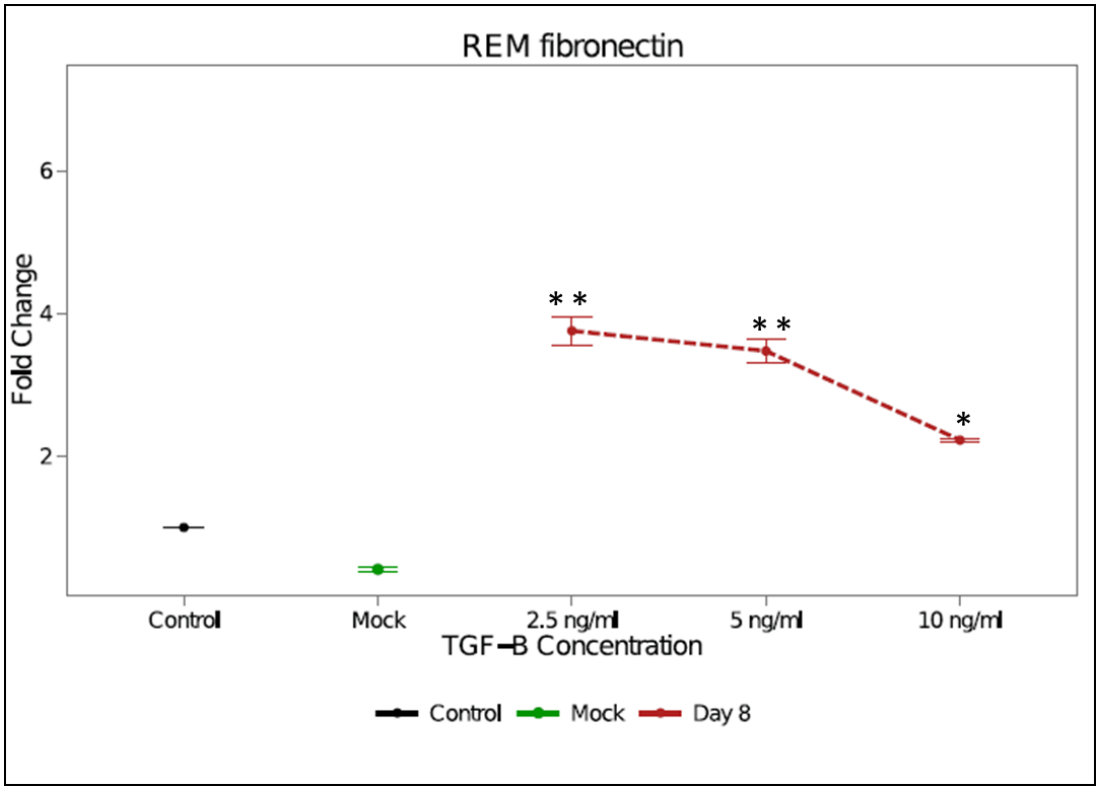

Figure 3.11. The mesenchymal marker fibronectin is highly upregulated in REM cells after TGF-β stimulation at day 8 compared to the control groups. Control = untreated cells, Mock = vehicle-treated cells for 8 days with the equivalent of 10 ng/ml of TGF-β, 2.5 ng/ml = cells stimulated with 2.5 ng/ml of TGF-β, 5 ng/ml = cells stimulated with 5 ng/ml of TGF-β, 10 ng/ml = cells stimulated with 10 ng/ml of TGF-β. Day 8 = cells harvested at day 8 of treatment. We found statistically significant differences in fibronectin expression between cells harvested after 8 days of treatment with TGF-β and control and vehicle-treated control cells. P values comparing TGF-β-stimulated samples with control cells: * = p value ≤ 0.05. ** = p value ≤ 0.005.

3.3.4 EMT confers migratory properties to feline and canine mammary carcinoma cell lines

The migration capacity of TGF-β-stimulated REM and CMC cells was assayed using a wound healing assay. REM and CMC were treated with 10 ng/ml of TGF-β for 6 days, prior to a scratch being made on the cell monolayer. To determine how quickly cells healed the gap, measurements were taken every 4 hours and the relative migration distance was calculated. TGF-β-stimulated REM and CMC cells showed a faster migration rate than untreated control cells.

TGF-β-stimulated REM cells migrated to close the scratch made (wound) after 32 hours of incubation, whereas non-stimulated cells took at least 48 hours to close the wound as shown in Figure 3.12.

The CMC cell line also showed a faster migration rate in TGF-β-stimulated cells compared to the untreated cells. In this case, stimulated cells closed the wound completely after 56 hours of incubation in contrast to untreated cells which closed less than 70% of the wound by that same time point as depicted in Figure 3.13.

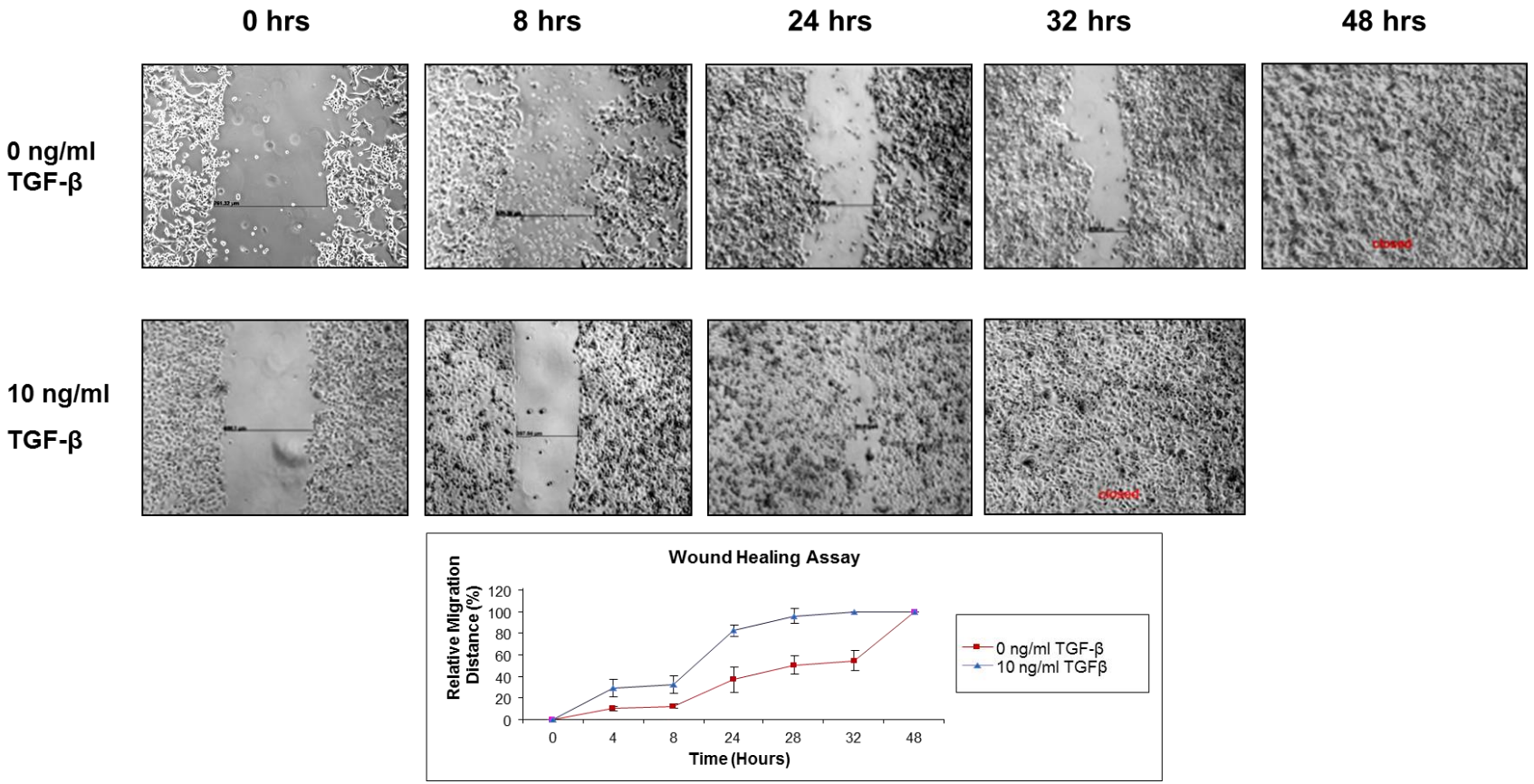

Figure 3.12. In vitro scratch assay of REM cell line showing the effect of TGF-β induced EMT on cellular migration. Images were taken at 0, 8, 24 and 32 hours for stimulated cells and a 48 hours image was included for untreated cells. Stimulated cells achieved 100% wound closure at 32 hours compared to untreated cells which relative migration distance at this time point was less than 60%. In the graph, each bar represents the mean (SD) of the measurements in triplicates. 0 ng/ml TGF-β = untreated cells, 10 ng/ml TGF-β = cells stimulated with 10 ng/ml of TGF-β.

Figure 3.13. In vitro scratch assay of CMC cell line showing effect of TGF-β induced EMT on cellular migration. Images were taken at 0, 12, 24 and 56 hours. Stimulated cells achieved 100% wound closure at 56 hours compared to untreated cells which relative migration distance at this time point was less than 70%. Each bar represents the mean (SD) of the measurements in triplicates. 0 ng/ml TGF-β = untreated cells, 10 ng/ml TGF-β = cells stimulated with 10 ng/ml of TGF-β

3.4 Discussion

In companion animals type III EMT has not been widely studied. Different research groups have found important results regarding type II EMT (wound healing and fibrosis) (Aresu et al., 2007, Chandler et al., 2007, Aresu et al., 2008, Mathias et al., 2009). Other studies have correlated tumour progression with the expression of adhesion molecules and EMT-associated transcription factors (Mcentee and Brennerman, 1999, Nowac et al., 2007, Nowac et al., 2008, Aresu et al., 2010, Han et al., 2010, Stein et al., 2011, Ide et al., 2011, Im et al., 2012) but, interestingly, none of them have correlated their findings with type III EMT. Only few studies, including papers published by Salgado et al. and Fonseca-Alves et al., report findings regarding EMT in companion animals, related to cancer progression. These papers show an important correlation between EMT markers and mammary and prostatic carcinoma progression in dogs, as described in Chapter 1.16 (Salgado et al., 2014 and Fonseca-Alves et al., 2015).

Here, we observed that canine, feline and human mammary carcinoma cells undergo an EMT after stimulation with TGF-β and show a more mesenchymal phenotype. These mesenchymal-like characteristics were demonstrated by morphological and molecular changes. E-cadherin protein expression was repressed whilst there was increased protein expression of mesenchymal markers. Previous studies have shown that upregulation of mesenchymal markers is a consequence of repression of E-cadherin (reviewed by Thiery and Sleeman, 2006). Here we included the human carcinoma cell line MCF7 as a comparative control.

We have demonstrated that dog and cat mammary carcinoma cells undergo EMT following TGF-β stimulation and consequently show morphology associated with mesenchymal cells including spindle shapes; loss of cell-cell contact; and separation from the group of cells. It took at least 6 days of TGF-β stimulation at a concentration of 10 ng/ml every 24 hours for maximal morphological changes to be observed. These results were consistent in all three cell lines studied. TGF-β is a complex molecule with multifaceted and context-dependent effects on cell function. For example, as described in Chapter 1.10, in early stages of cancer, TGF-β can act

as a tumour suppressor gene, whereas at late stages it can act as a tumour promoter (Shipitsin et al., 2007), enhancing cancer progression and metastasis through an EMT (Levy and Hill, 2005).

The REM and CMC cells, after TGF-β treatment, showed a more spindle shape, especially near the edges of the cell group. MCF7 cells also showed similar changes in morphology, in which the cells became not only longer but also wider, although these changes were quite limited to the edges of the groups of cells. These morphological changes would be indicative that these cells are going through an EMT process as reviewed by *Thiery, 2003*. Further to this, protein expression of molecular markers of EMT was analysed. Epithelial and mesenchymal markers showed a decrease and increase in protein expression, respectively. These changes were present at a similar time following TGF-β stimulation to the morphological changes associated with an EMT process. These findings were also observed in all three cell lines. Several studies have shown the inverse correlation that transcription factors ZEB1 and Twist have with E-cadherin expression during EMT-induced metastasis. Eger et al, 2005 showed that human breast cancer cells expressing high RNA and protein levels of ZEB1 also expressed low levels of E-cadherin and were not able to form complete epithelial sheets. They also knocked down ZEB1 and observed upregulation of E-cadherin, confirming that ZEB1 is an important repressor of E-cadherin and thus, might play an important role in invasion and metastasis of epithelial cancer cells (Eger et al., 2005). In a similar way, *Yang et al, 2004* demonstrated the relevance of Twist during EMT in epithelial cancer cells including breast cancer in humans. They utilised siRNAs to knockdown Twist and observed how breast cancer cells had an inverse correlation between Twist and E-cadherin expressions, as the knocked-down cells increased their expression of E-cadherin and reduced their migratory abilities. Furthermore, they confirmed that Twist is an E-cadherin suppressor and also induces mesenchymal markers, such as fibronectin and vimentin (Yang et al., 2004).

In the current study, we showed that CMC, a feline mammary carcinoma cell line also showed increased expression of transcription factor ZEB1, which could play an important role in type III EMT in feline mammary cancer. Similarly, the REM

canine cell line showed overexpression of the transcription factor Twist after TGF-β stimulation. These findings could lead to new understanding regarding potential effectors of type III EMT in companion animals, although there is still much to be understood about the diverse transcription factors and their roles in EMT, tumour progression and metastasis. Similar experiments would have to be carried out using *in vivo* models such as the chorioallantoic membrane (CAM) assay to further explore these findings and assess the effects of targeting these transcription factors on progression and metastasis. Moreover, in order to demonstrate if the effects of ZEB1 and Twist are crucial for E-cadherin expression during EMT in companion animals, it would be of great importance to analyse whether silencing these transcription factors enhance E-cadherin expression or not.

At the transcription level, EMT associated changes were observed by qRT-PCR where MCF7 cells showed a downregulation of epithelial markers E-cadherin and β-catenin after 8 days of TGF-β treatment while the mesenchymal marker vimentin was upregulated in a dose dependent manner with TGF-β after stimulation for 4 and 8 days. Interestingly, after only 4 days of TGF-β treatment, these cells demonstrated increased expression of the above mentioned epithelial markers (data not shown). This could be consistent with the variable and context-specific effects that TGF-β induces, as mentioned in Chapter 1.10 and described by (Lehmann et al., 2000, Levy and Hill, 2005 and Shipitsin et al., 2007). If we compare these results with our MCF7 morphological data, we can assume that these cells need between 6 and 8 days of treatment with TGF-β in order to acquire both morphological and molecular changes associated with EMT. This gives evidence that MCF7 cells go through a TGF-β-induced EMT losing their epithelial characteristics and acquiring mesenchymal traits after TGF-β stimulation. In contrast to the MCF7 cell line, the REM cell line did not show an evident downregulation of epithelial markers after TGF-β stimulation, although, the mesenchymal marker fibronectin was highly upregulated in TGF-β-stimulated cells after 8 days with all concentrations of TGF-β. While these REM cells acquired mesenchymal properties, they did not show loss of epithelial characteristics at the transcription level. This finding is discordant with the morphological changes associated with loss of epithelial characteristics that we

observed after 6 days of TGF-β stimulation in these same cells. Nevertheless, as mentioned in Chapter 3.2, in some cases, cells may pass through a partial EMT without showing an evident downregulation of epithelial markers or upregulation of mesenchymal markers, making invading cells phenotypically similar to either an epithelial or a mesenchymal cell (Thompson et al., 2005, reviewed by Tarin et al., 2005, and Yang and Weinberg, 2008). This could be an explanation as to why canine mammary carcinoma cells did not show an evident downregulation of epithelial markers while fibronectin was clearly upregulated after TGF-β stimulation. Furthermore, there is disparity between epithelial markers at the mRNA and protein levels in REM and MCF7 samples after TGF-β stimulation. This could be happening because even though the mRNA expression levels may act as a predictive outcome for translational activity, in some cases mRNA expression levels only partially predict corresponding protein levels (reviewed by Vogel and Marcotte, 2013). A different explanation of these discrepancies is that regulation by different mechanisms, including microRNAs, at the post-transcriptional or translational level fine-tunes protein expression (Baek, et al., 2008). This can be related to feedback loops that can regulate and integrate transcription, translation and degradation levels as reviewed by *Dahan et al., 2011*. Along with this, Laurent et al, (2010) showed that protein levels can be more conserved than their corresponding mRNA levels in different cells, including bacteria, yeast, human and plants (Laurent, et al., 2010). In line with these observations, in this study we focussed our attention more on the post-transcriptional levels of protein expression than in transcriptional levels of mRNA, as it closely correlates to the observed morphological changes and migratory capability of EMT-induced mammary carcinoma cells.

As previously mentioned in Chapter 1.5, prior to undergoing an EMT, epithelial cells usually remain attached to their basement membrane with a top to bottom polarity which prevents them from moving through surrounding tissues as reviewed by *Acloque et al., 2009*. Once they undergo an EMT, they acquire migratory features as their polarity is changing to a front to back polarity with decreased adherence junctions, enabling them to move more easily and rapidly (reviewed by Savagner et al., 1994, Thiery and Chopin, 1999 and Acloque et al.,

2009). This migratory characteristic can be assessed by different means. Motility is one of the most important characteristics of epithelial cells undergoing a type II EMT in which they would need to migrate in order to colonise the wound which induced type II EMT and heal the tissue, or perhaps promote fibrosis. In this study, we assessed how fast mammary carcinoma cells from dogs and cats migrated to colonise an artificial wound *in vitro* with and without exposure to TGF-β. Cells treated with TGF-β migrated faster than untreated cells, colonising the wound at least 16 hours prior to their untreated counterpart. These observations confirm that EMT gives migratory characteristics to feline and canine mammary carcinoma cells and that TGF-β is a promoter of this process *in vitro*. Other research groups have shown similar results *in vivo*, utilising gastric carcinoma cells pre-treated with TGF-β and injected into the abdominal cavities of nude mice, where they observed that treated cells exhibited higher liver metastatic rates than untreated cells (Fu et al., 2009). Although these characteristics have not been studied in dogs and cats *in vivo*, they suggest avenues for future research in this field.

In conclusion, there is still much to be understood in cancer research; and tumour progression and metastasis are some of the most important parts of this field. Our results could be further explored with different approaches to continue research in this field. For instance, confirmation of some crucial points associated with our findings by inhibiting different TGF-β-associated transcription factors with specific siRNAs in order to better understand their roles in EMT. Targeted therapies against specific molecular receptors or signalling pathways associated with EMT are regarded as important tools to prevent cancer progression, as reviewed by *Davis et al., 2014*; and if the inhibition of these molecules proves useful *in vitro*, this could direct the rational development of targeted drugs against the salient molecules within the TGF-β pathway, leading ultimately to clinical trials. Small animals (dogs and cats) can be utilised as good comparative models for the study of cancer in humans (Vail and Macewen, 2000, reviewed by Porrello et al., 2006, Boggs et al., 2008, Gama et al., 2008, Cassali et al., 2011). Most of the studies we have been carrying out have been already demonstrated in human cell lines with similar outcomes, thus, these similarities can be used for the development of novel techniques and

experimental protocols. Perhaps the similarities in various types of cancer and its progression in humans and companion animals are due to the fact that cancers in dogs, cats and humans occur spontaneously compared to artificially-induced rodent models (Hansen and Khanna, 2004, Allred and Medina, 2008 and reviewed by Burrai et al., 2010, and Printz, 2011). Some specific examples of the similarities between human and canine cancers include mammary, prostate, lung and bladder carcinomas among other types of sarcomas and lymphomas as reviewed by *Porrello et al., 2006.* Interestingly, *Bonnomet et al., 2012,* developed and characterised genetically engineered mouse models in which they transplanted human breast tumours and assessed EMT-associated changes similar to those in human cancer. These changes included upregulation of mesenchymal markers such as vimentin, snail and slug, and downregulation of E-cadherin among other epithelial markers. However, these laboratory animal recipients need to be genetically modified in order to be able to receive a xenotransplant and develop the specific tumour characteristics, unlike companion animals which can spontaneously develop cancer, as reviewed by *Printz, 2011*. It has been suggested that despite the wide variety of mouse models to study human prostate cancer (Wang, 2011) it would be impossible to reproduce all characteristics of such disease in mice as reviewed by *Hensley and Kyprianou, 2012*. The anatomy and physiology of the prostatic gland in humans and dogs share many similarities (Leroy and Northrup, 2009) that could explain the observations of *Keller et al*, that dogs are the only known large mammals to spontaneously exhibit all stages of prostatic disease progression from benign hyperplasia to an invasive, metastatic carcinoma as in humans (Keller et al., 2013). *Suami et al* demonstrated the similarities between the lymphatic systems of dogs and humans (Suami et al., 2013), an important feature to trace cancer progression and the metastatic potential of specific tumours. In line with these observations, it would be of great relevance to our future research to assess the similarities in expression of EMT-associated markers in lymphatic metastases in humans and dogs with mammary carcinoma. A study carried out in 2014 showed that loss of E-cadherin in mammary carcinoma primary tumours in humans was related to the acquisition of a mesenchymal phenotype in 45% of circulating tumour cells from the same patients, compared with only 23% of circulating tumour cells showing a mesenchymal phenotype in patients

with tumours that did not show a decrease in E-cadherin expression by immunohistochemistry. When comparing primary tumours with metastatic lymph nodes, they found upregulation of EMT-associated transcription factors, but re-expression of E-cadherin with lower cell division rates (Markiewicz et al., 2014). Correlation of our findings in primary tumour cell lines with metastatic cell lines could reveal the salient EMT factors involved in successful metastasis.

Other molecular similarities between dogs and humans with regard to cancer progression include tyrosine kinase receptors (TKRs). Tyrosine kinase receptors are involved in tumour proliferation and angiogenesis as reviewed by *Ranieri et al., 2014*. The epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptor (PDGFR), stem cell factor receptor (c-KitR), and colony-stimulating factor 1 (CSF-1) are TKRs, which have been identified in the tumours of both species (reviewed by Shchemelinin et al., 2006, Buchdunger et al., 2000, Nakopoulou et al., 2002) . It has been proposed that cell proliferation in cancer can be due to alterations in the phosphorylation of protein kinases after aberrant activity or deregulation of these receptors (reviewed by Cohen, 2002, London et al., 2003). These findings enlighten the possibility of creating novel selective and personalised therapeutics against cancer and its progression in humans and pets by using this type of TKRs as rational targets for the development of tyrosine kinase inhibitors (TKIs) for both species (Krause and Van Etten, 2005, London et al., 2009). It has been proposed that among these TKRs, EGFR is able to regulate cell migration and invasion in malignant epithelial cells through an EMT (reviewed by Al Moustafa et al., 2012). Thus, TKIs which could potentially block EGFR pathways might be able to inhibit cell migration and invasion, which would make them important components for combating cancer metastasis

Elucidating the potential roles of several EMT-associated transcription factors in different molecular pathways can lead to a more detailed understanding of type III EMT and can help guide the rational development of new diagnostic and therapeutic tools for cancer and its progression.

Chapter 4: Epithelial to Mesenchymal Transition generates cancer cells with stem cell characteristics

4.1 Abstract

Induction of EMT can produce cells with stem cell-like characteristics, including self-renewal and resistance to chemotherapeutic drugs and apoptosis. This gives rise to many questions in the process of metastasis and tumour progression. Cells acquiring migratory and invasive properties can also become resistant and can become able to colonise a new niche and self-renew in order to produce progeny and create micrometastases which can develop into macroscopic secondary masses. Here, we have demonstrated the ability of breast cancer cells in dogs and cats to acquire stem cell-like characteristics by TGF-β-induced EMT. After TGF-β-induced EMT cells showed a better ability to generate spheres, which are representative of CSCs, while they showed a more mesenchymal phenotype analysed by epithelial and mesenchymal markers at the protein expression level. TGF-β-stimulated cells also showed enhanced resistance to doxorubicin compared to untreated cells.

4.2 Introduction

Recent studies have shown that tumours are made up of a heterogeneous mix of cells that consists of a small CSC population and a bulk population of more differentiated cells as reviewed by *Reya et al., 2001*. The cells with stem cell characteristics usually comprise a very low percentage of the tumour mass and some of their properties include constant self-renewal and acquisition of resistance to both apoptosis and therapeutic modalities including chemotherapy and radiotherapy (reviewed by Dean et al., 2005, Diehn and Clarke, 2006, Phillips et al., 2006, Pang et al., 2013). One important aspect of these CSCs is that they are more capable of colonising a new microenvironment after cell migration and developing a metastatic tumour than regular cancer cells. This observation was confirmed by *Nomura et al., 2015*, after they showed in an athymic mouse model that the tumourigenicity of injected cultured human pancreatic carcinoma cells was enhanced by ectopic overexpression of CD133 (a stem cell marker) compared to the control, empty plasmid (Nomura et al., 2015). After arriving at a different organ, these CSCs can colonise it and differentiate so they can produce a new tumour in their new niche (reviewed by Dave et al., 2012 and reviewed by Dean et al., 2005).

Tumour-seeding ability and resistance to apoptosis and antineoplastic properties is usually exhibited by stem cells and can be acquired by epithelial cancer cells after a TGF-β-induced EMT as shown by *Mani et al* (Mani et al., 2008). They demonstrated that TGF-β was able to induce EMT in human mammary epithelial cells (HMECs), which was confirmed by EMT-associated changes, whereby epithelial markers were downregulated and mesenchymal markers were upregulated. These EMT-induced cells were expressing the stem cell markers CD44+CD24-. The expression of these cell surface markers is characteristic in epithelial stem cells and human breast cancer cells with stem cell-like characteristics (Al-Hajj et al., 2003, Mani et al., 2008).

A number of studies have confirmed the tumorigenic ability of epithelial breast cancer cells acquired after an EMT process. *Morel et al* (Morel et al., 2008) observed that a human mammary oncogenic cell line (HMLER), which expressed

CD44+CD24- by FACS, was able to form mammospheres, which are considered to be tumorigenic. These characteristics are associated with stem cells. They also found that the CD44+CD24- fraction in HMEC and MCF10 (immortal human mammary epithelial cell line) cell lines showed EMT associated changes at the protein expression level, suggesting that there could be a link between EMT and the acquisition of stem cell characteristics. They also induced EMT in CD44-CD24+ MCF10 and HMEC cell lines by treating them with TGF-β for 8 days and observed that mesenchymal markers were upregulated whilst epithelial markers were downregulated. Moreover, after EMT induction, small subpopulations of these cell lines expressed CD44+CD24- cell surface markers, compatible with cells with stem cell-like characteristics (Morel et al., 2008).

Furthermore, other studies have confirmed the expression of the stem cell surface marker CD133 in breast cancer cells. *Xiao et al* observed that CD133+ human inflammatory breast cancer (IBC) cells have the ability to from spheroidal cells under limiting survival conditions with serum-free growth medium, suggesting that CD133+ cells have stem cell-like characteristics including self-renewal and resistance to limiting survival conditions (Xiao et al., 2008). In line with these observations, another research group have found that after an induced EMT human epithelial breast cancer cells acquire stem cell-like characteristics and express CD133. They observed that at least 50% of patients with breast cancer expressing CD133 had tumour relapse after chemotherapy, consistent with CD133+ breast cancer cells being not only resistant to limiting survival conditions , but also resistant to the effects of chemotherapeutic agents (Nadal et al., 2013). In line with these observations, the expression of stem cell surface markers are of vital importance for the identification and isolation of putative CSCs. Moreover, these cell surface markers can be utilised to further validate that epithelial cancer cells can become CSCs after induction of EMT.

In one study by *Li et al in 2008*, the subpopulation of tumour-initiating cells identified in human breast cancer was found to be more resistant to conventional therapies than the more differentiated cancer cells (Li et al., 2008). The implication is that tumour relapse can be accomplished by these perseverant tumour-initiating cells

after conventional treatment. Therefore, CSCs must be eliminated to affect a cure on cancer.

Chemotherapy is more effective against tumours with high growth fraction (GF) and low mass doubling time (MDT) as most chemotherapeutic agents act upon the process of cell division. Most tumours have a shorter MDT and higher GF during early stages of tumour growth, thus, chemotherapeutic agents usually work better at early stages. Chemotherapy is not very efficient in large tumours, as they would already be in late stages where the GF is lower and the MDT is longer, but metastatic lesions are potentially more susceptible as they have shorter MDT and higher GF than the primary tumour as reviewed by *Argyle, 2008*. Even though there are no clinical studies confirming their efficacy, two of the most utilised chemotherapeutic agents in small animal breast cancer are doxorubicin and mitoxantrone (Novosad, 2003, Gimenez et al., 2010, Clemente et al., 2009, Clemente et al., 2010). Doxorubicin is an antineoplastic antibiotic which can be used alone or in combination with other agents. It inhibits DNA, RNA and protein synthesis, throughout the whole cell cycle, although the exact mechanism of action is unknown (Plumb, 2005). Mitoxantrone is an antineoplastic drug which binds to DNA and inhibits DNA and RNA synthesis. It does not act in a specific phase of the cell cycle, but it appears to be more active during the S phase (Plumb, 2005). Cancer cells with stem cell-like characteristics have been proven to be more resistant to apoptosis and to conventional therapies, thus, finding new ways to target them is needed in order to treat cancer and prevent its progression and tumour relapse (reviewed Dave et al., 2012, Phillips et al., 2006 and reviewed by Dean et al., 2005). Targeting EMT inducers may offer a novel way to treat cancer patients as it has been demonstrated by different research groups that EMT-induced cancer cells exhibit stem cell-like characteristics (Mani et al., 2008, Morel et al., 2008, reviewed by Polyak and Weinberg, 2009 and Blacking et al., 2007). The development of new therapies blocking EMT and its transcription factors might be of great importance to provide an effective tool against cancer and its progression. In other words, if we confirm that there is indeed a crosstalk between EMT and the acquisition of stem cell-like characteristics in breast cancer, treatment of cancer at earlier stages with EMT-

blocking agents could inhibit EMT effectors including ZEB1 and Snail, as shown by *Olmeda et al, 2007a* (Olmeda et al., 2007a). If EMT were blocked, not only might migration of cancer cells be prevented, but also the acquisition of other stem cell-like characteristics. Such inhibition of self-renewal and resistance to apoptosis and chemotherapeutic agents, could retard or arrest development of primary and metastatic tumours by decreasing cancer cells multiplication and augmenting the efficacy of currently used therapies.

In this study, we have demonstrated that mammary carcinoma cells from dogs and cats acquired tumorigenic characteristics after TGF-β-induced EMT. Our data is consistent with other research groups working on mammary epithelial and mammary carcinoma cells in humans (Mani et al., 2008, Morel et al., 2008, and reviewed by Polyak and Weinberg, 2009). In these studies, TGF-β-stimulated epithelial mammary cells were able to form spheres and expressed stem cell associated markers, suggesting a link between EMT and the acquisition of stem cell characteristics in breast cancer cells. By confirming the acquisition of tumorigenic traits in canine and feline TGF-β-stimulated breast cancer cells, herein we could establish a link between EMT and the acquisition of stem cell-like characteristics in companion animals. These findings are significant in cancer research in veterinary medicine as studies establishing a link between EMT and CSCs have not been previously reported. Our findings also further support dogs and cats as important animal models for human breast cancer research.

4.3 Results

4.3.1 TGF-β-stimulated mammary carcinoma cells have increased sphere forming ability

Canine and feline mammary carcinoma cells showed an increased ability to form spheres after TGF-β stimulation. Cells were treated with 10 ng/ml of TGF-β for 6 days before seeding them in N2 media (without serum and low levels of glucose) in low-adherent plates as described in Chapter 2.4. We observed that TGF-β-stimulated feline and canine mammary carcinoma cells were able to form more spheres than vehicle-treated cells. Feline mammary carcinoma spheres were bigger in size than the few spheres formed by unstimulated cells as shown in Figure 4.1. On average, TGF-β-stimulated feline mammary carcinoma cells formed approximately 44 spheres per microscopic field (objective 10x), whereas untreated cells formed approximately 16, shown in Figure 4.2. Moreover, to assess if spheres had a mesenchymal phenotype, we assessed the expression of epithelial and mesenchymal protein markers by western blotting. The concentrations of mesenchymal markers were upregulated in non-TGF-β-stimulated spheres compared to adherent cells, consistent with there being a correlation between the acquisition of stem cell-like characteristics and EMT as depicted in Figure 4.3.

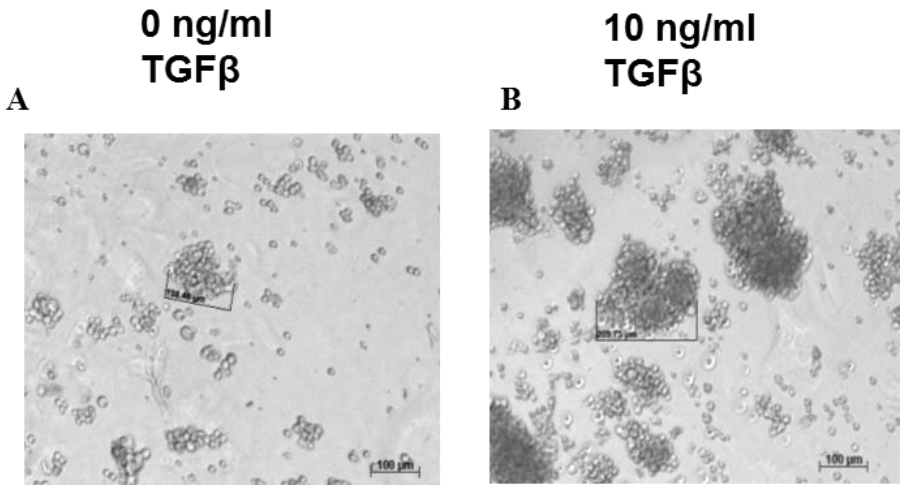

Figure 4.1. Spheres derived from feline mammary carcinoma cell line (CMC) treated with 10 ng/ml of TGF-β for 6 days are larger than spheres derived from untreated cells. A) Untreated CMC cells. B) TGF-β-stimulated CMC cells. Scale bar represents 100 µm.

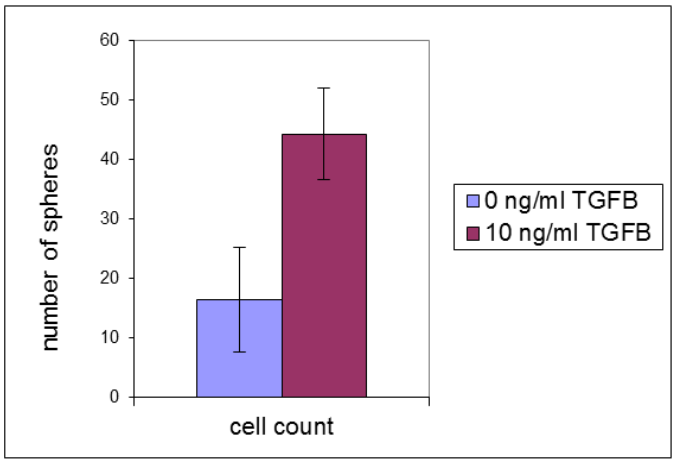

Figure 4.2. TGF-β-stimulated feline mammary carcinoma cell line (CMC) demonstrate greater sphere forming ability compared with untreated cells. Cells were stimulated with 10 ng/ml of TGF-β for 6 days and formed an overall average of 44 spheres per 10 x objective field, compared to the untreated cells, which formed an average of 16 viable spheres. Each bar represents the mean of the average measurements of 6 counts, whiskers represent the SD ($p < 0.005$, Mann-Whitney test).

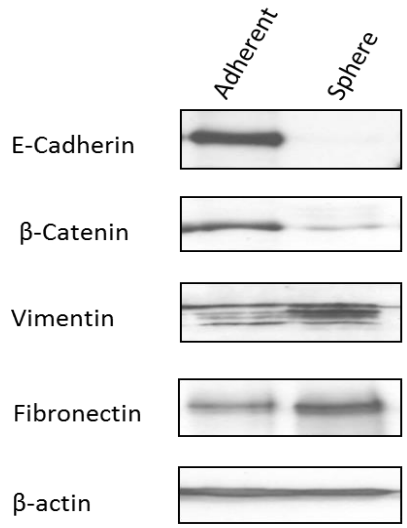

Figure 4.3. CMC spheres display a mesenchymal phenotype at the protein expression level. When compared to adherent cells, non-TGF-β-stimulated spheres express higher levels of mesenchymal markers vimentin and fibronectin, and lower levels of epithelial markers E-cadherin and β-catenin, associated with a mesenchymal phenotype. Image courtesy of Dr. Lisa Pang. β-actin was used as a loading control for all cell samples. All proteins were expressed at the expected molecular weights, where E-cadherin = 120 kDa, β-catenin = 92 kDa, vimentin = 57 kDa, fibronectin = 240 kDa and β-actin = 45 kDa. Thirty µg of protein was loaded for each immunoblot.

The canine mammary carcinoma cell line (REM) also showed increased sphere forming ability after TGF-β stimulation. TGF-β-stimulated cells formed more and up to 10 times larger spheres than the untreated group as shown in Figure 4.4 and Figure 4.5. Treated cells produced an overall average count of 37 spheres per 10 x microscopic field, compared to the untreated cells, which formed an average count of 10 spheres per 10 x field. Furthermore, similar to our observations in feline mammary carcinoma spheres, we confirmed that canine mammary carcinoma non-TGF-β-stimulated spheres acquired a mesenchymal phenotype with higher expression of mesenchymal markers while epithelial markers were downregulated compared to adherent REM cells as depicted in Figure 4.6.

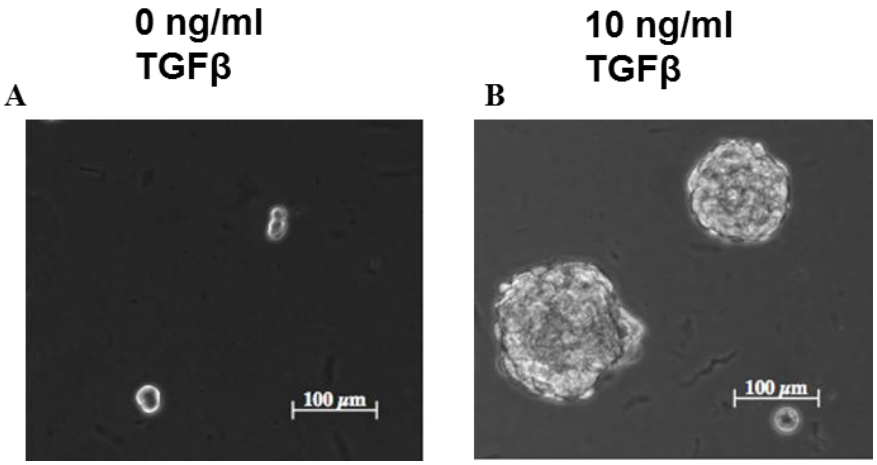

Figure 4.4. REM cells acquire enhanced sphere forming abilities after TGF-β stimulation. Canine mammary carcinoma cells were treated with 10 ng/ml of TGF-β for 6 days and formed an increased number and larger spheres than untreated cells A) Untreated REM cells. B) TGF-β-stimulated REM cells. Scale bar represents 100 µm. Image courtesy of Dr. Lisa Pang.

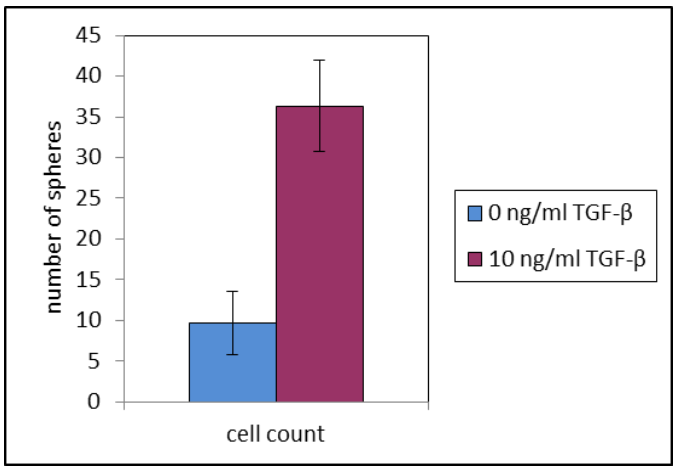

Figure 4.5. REM cells show better sphere forming ability in TGF-β-stimulated cells, compared with untreated cells. Cells stimulated with 10 ng/ml of TGF-β for 6 days formed an overall average count of 37 spheres per 10 x field, compared to the untreated cells, which formed an average count of 10 spheres per 10 x field. Each bar represents the mean of the average measurements of 6 counts, whiskers represent SD (p = 0.005, Mann–Whitney test).

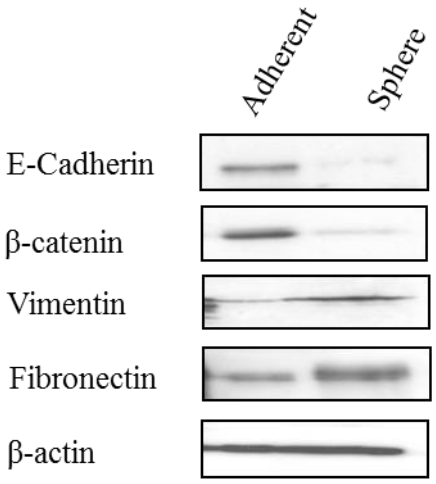

Figure 4.6. REM spheres display a mesenchymal phenotype at the protein expression level. When compared to adherent cells, non-TGF-β-stimulated spheres express higher levels of mesenchymal markers vimentin and fibronectin and lower levels of epithelial markers E-cadherin and β-catenin, associated with a mesenchymal phenotype. B-actin was used as a loading control for all cell samples. All proteins were expressed at the expected molecular weights, where E-cadherin = 120 kDa, β-catenin = 92 kDa, vimentin = 57 kDa, fibronectin = 240 kDa and β-actin = 45 kDa. Thirty µg of protein was loaded for each immunoblot. Image courtesy of Dr. Lisa Pang.

Here we have shown that induction of EMT by TGF-β in both canine and feline mammary carcinoma cells leads to an increased ability to form spheres in low adherence conditions, which is a CSC characteristic. Spheres also have high expression of mesenchymal markers and low expression of epithelial markers. Together these data supports a link between TGF-β induction of EMT and the acquisition of CSC characteristics.

4.3.2 TGF-β-stimulated canine and feline mammary carcinoma cell lines are enriched for cells expressing the stem cell marker CD133

To assess if TGF-β treatment elevated the number of cells expressing the CSC marker CD133, we treated REM and CMC cells with 10 ng/ml of TGF-β for 6 days as described in Chapter 2.3.1, then sorted by magnetic activated cell sorting (MACS), described in Chapter 2.10. Here we found that TGF-β treatment increased the percentage of CD133+ cells from 1.08% to 3% in REM cells and from 0.69% to 1.3% in CMC cells compared to vehicle-treated cells. These findings suggest that the number of REM cells with stem cell characteristics is approximately three times higher after TGF-β stimulation than in untreated cells as shown in Table 4.1. Similarly, we observed that in the CMC cell line, cells treated with TGF-β express the stem cell marker CD133 twice as frequently as untreated cells as shown in Table 4.2.

	CD133 +	CD133 -	Total
REM	1.08%	98.92%	100%
REM T	3%	97%	100%

Table 4.1. TGF-β-stimulated REM cells express a higher percentage of CD133+ cells than untreated cells. Treatment with 10 ng/ml of TGF-β for 6 days increases the percentage of CD133+ cells from 1.08% to 3%. REM T = REM cells treated with 10 ng/ml of TGF-β. REM = REM cells without treatment.

	CD133 +	CD133 -	Total
CMC	0.69%	99.30%	100%
CMC T	1.30%	98.70%	100%

Table 4.2. TGF-β-stimulated CMC cells express CD133 in a higher percentage than the untreated fraction. Treatment with 10 ng/ml of TGF-β for 6 days increases the percentage of CD133+ cells from 0.69% to 1.3%. CMC T = CMC cells treated with 10 ng/ml of TGF-β. CMC = CMC cells without treatment.

4.3.3 Breast cancer cells acquire resistance to chemotherapy after TGF-β stimulation

Canine and feline mammary carcinoma cells showed an enhanced sphere forming ability after TGF-β stimulation as shown in Chapter 4.3.1. To assess if these cells also acquired resistance to therapeutic modalities like chemotherapy, TGF-β-stimulated cells were treated with two different chemotherapeutic agents. The chemotherapeutic agents used were doxorubicin and mitoxantrone; both of which are commonly used to treat breast cancer in small animals. We observed that CMC cells did not show any significant difference in chemosensitivity between a TGF-β-stimulated cells and vehicle-treated cells. Both TGF-β-stimulated and unstimulated cells demonstrated a dose-response relationship with decreased viability with increasing concentrations of chemotherapy agent as shown in Figure 4.7 and Figure 4.8.

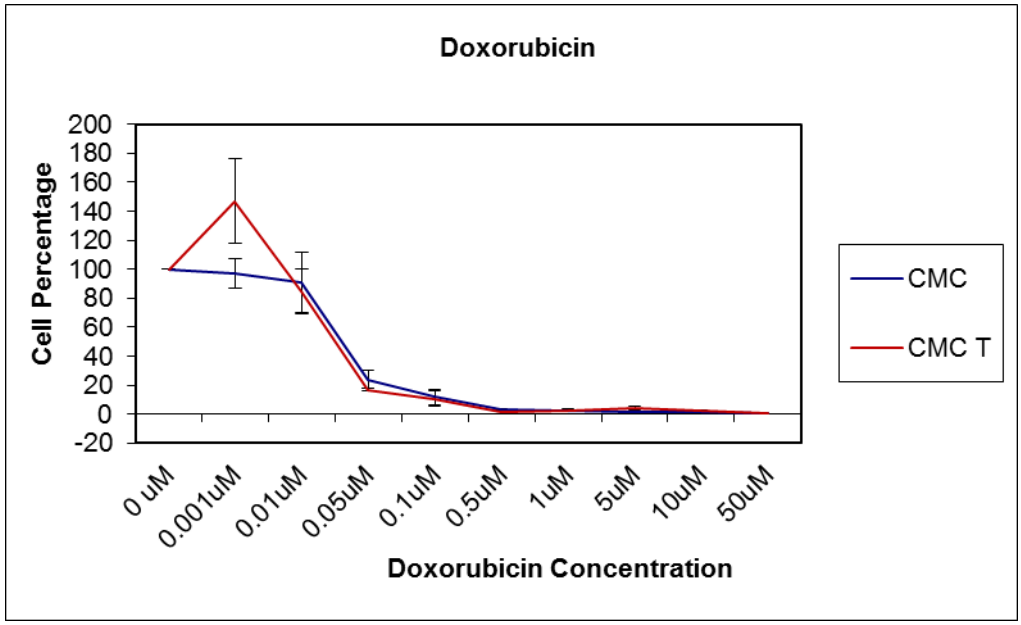

Figure 4.7. CMC cell survival showed a dose dependency with increasing concentrations of doxorubicin. TGF-β-stimulated cells did not show any difference in chemosensitivity compared to untreated cells over a range of concentrations of doxorubicin. A Mann-Whitney test was carried out, where the overall p-value = 0.7787. CMC = Vehicle-treated, control feline mammary carcinoma cell line; CMCT = TGF-β-stimulated CMC

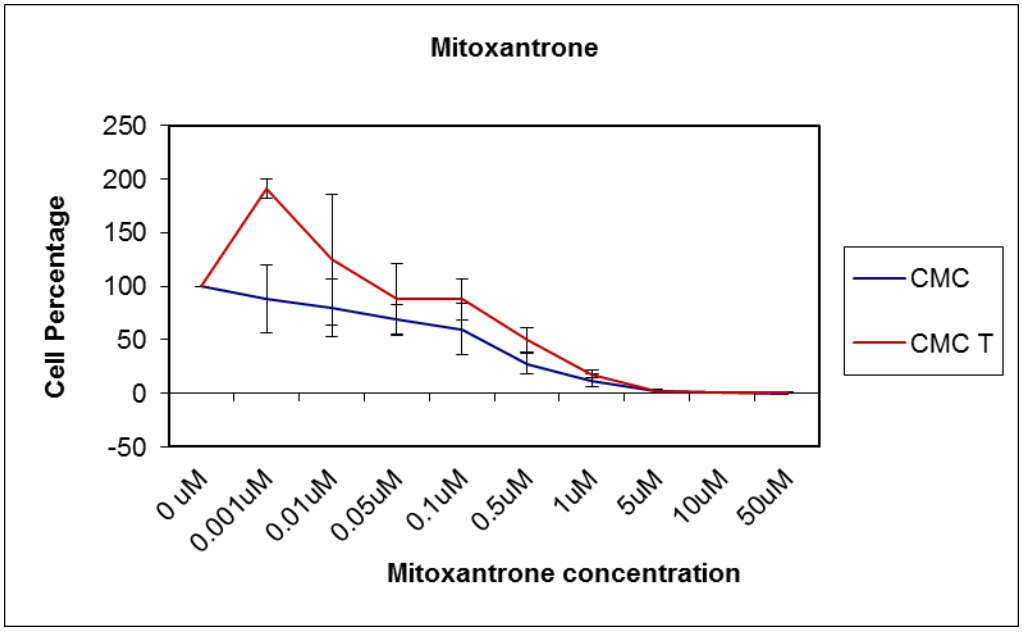

Figure 4.8. CMC cell survival showed dose dependency with increasing concentrations of mitoxantrone. TGF-β-stimulated cells did not show any significant difference in chemosensitivity compared to untreated cells over a range of concentrations of mitoxantrone. A Mann-Whitney test was carried out, where the overall p-value = 0.2085. CMC = Vehicle-treated, control feline mammary carcinoma cell line; CMCT = TGF-β-stimulated CMC

Unlike feline mammary carcinoma cells, TGF-β-stimulated REM cells showed enhanced resistance to doxorubicin in a chemosensitivity assay compared to vehicle-treated cells as shown in Figure 4.9. There was no significant difference in chemosensitivity between TGF-β-stimulated and vehicle-treated REM cells when treated with mitoxantrone (data not shown).

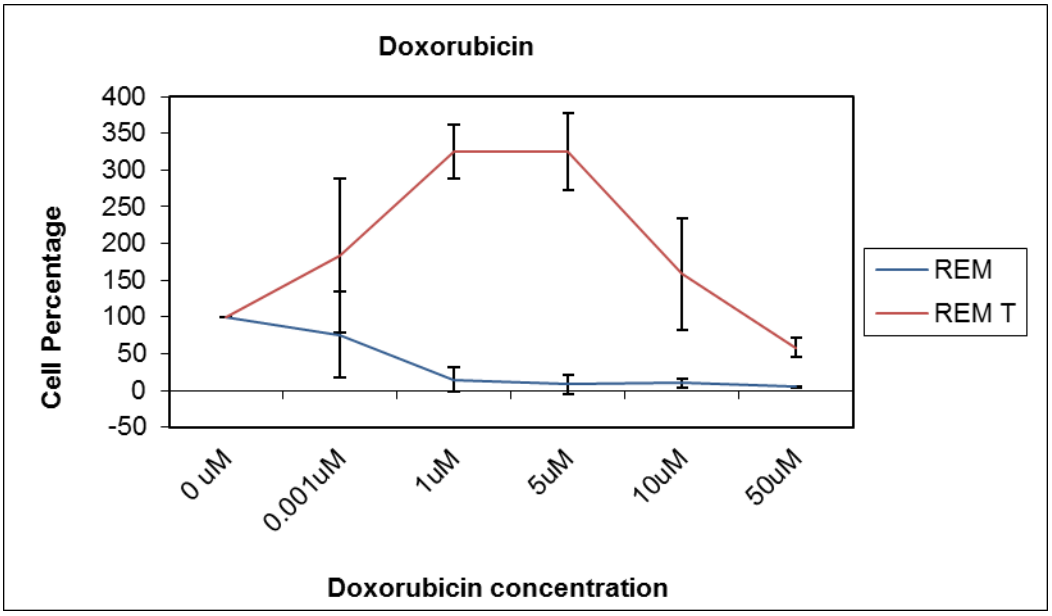

Figure 4.9REM cells treated with TGF-β are less sensitive to doxorubicin than are vehicle-treated control cells. REM cells stimulated with 10 ng/ml of TGF-β for 6 days prior to doxorubicin exposure showed enhanced chemoresistance, compared to vehicle-treated, control REM cells. REM = Vehicle-treated, control canine mammary carcinoma cell line; REMT = TGF-β-stimulated REM. A Mann-Whitney test was carried out where the overall p-value was 0.000002 for the whole experiment. An ANOVA test was carried out for the comparison between the untreated and treated groups at individual concentrations where p-values were 0.1067 for 0.001 µM of TGF-β, 0.00000007 for 1µM, 0.0000003 for 5µM, 0.0052 for 10µM, and 0.9081 for 50µM.

Furthermore, we utilised MACS to separate the CD133+ and CD133- fractions in REM cells. Untreated REM cells expressing CD133 showed greater resistance to doxorubicin compared to the CD133- fraction as depicted in Figure 4.10. As stated in Chapter 4.2, CD133 is a stem cell surface marker in some models, and cells expressing such validated stem cell markers would be expected to show greater chemoresistance than those lacking expression.

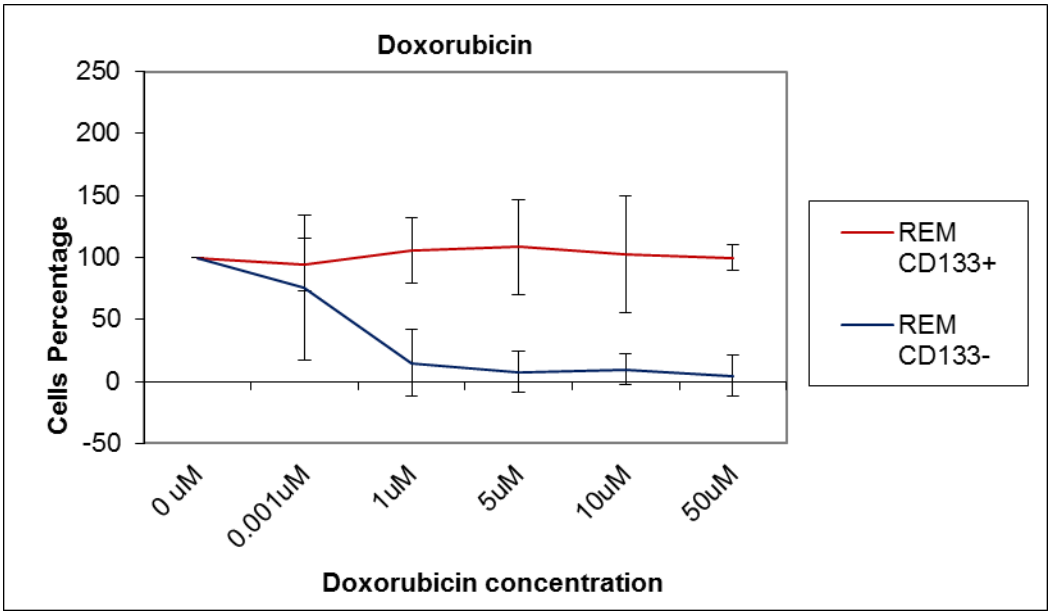

Figure 4.10. REM cells expressing CD133 are less sensitive to doxorubicin than are CD133- cells. REM CD133+ cells showed enhanced chemoresistance compared to CD133- cells. REM CD133+ = CD133+ REM cells; REM CD133- = CD133- REM cells. A Mann-Whitney test was carried out where the overall p-value was 0.00012 for the whole experiment. An ANOVA test was carried out for the comparison between the CD133+ and CD133- fractions at individual concentrations where p-values were 0.999 for 0.001 µM of TGF-β, 0.9551 for 1µM, 0.000884 for 5µM, 0.0335 for 10µM, and 0.0110 for 50µM.

Moreover, whether TGF-β-stimulated or unstimulated, sorted CD133+ REM cells exhibited resistance to increasing concentrations of doxorubicin. CD133+ cells sorted from TGF-β-stimulated REM cells showed no greater resistance to doxorubicin compared to CD133+ cells sorted from untreated REM cells, as shown in Figure 4.11. This finding suggests that cells expressing this stem cell surface marker exhibit the chemoresistance characteristic of stem cell-like cells. Interestingly, REM cells survived at increasing concentrations of the same chemotherapeutic agent after being treated with TGF-β, even when they did not express CD133 as shown in Figure 4.12. A possible explanation for these results could be that there might be a subpopulation of cells that do not express CD133 but still have stem cell-like characteristics including resistance to apoptosis and chemotherapeutic agents.

These data suggest that canine mammary carcinoma cells expressing CD133 are resistant to the cytotoxic effects produced by the chemotherapeutic drug doxorubicin regardless of TGF-β stimulation. Remarkably, cells previously stimulated with 10 ng/ml of TGF-β for 6 days showed resistance to doxorubicin even without expressing CD133, making them comparable to the CD133+ fraction. These results confirm that TGF-β might enable cells to acquire stem cell-like characteristics such as resistance to chemotherapeutic agents without necessarily expressing stem cell markers like CD133.

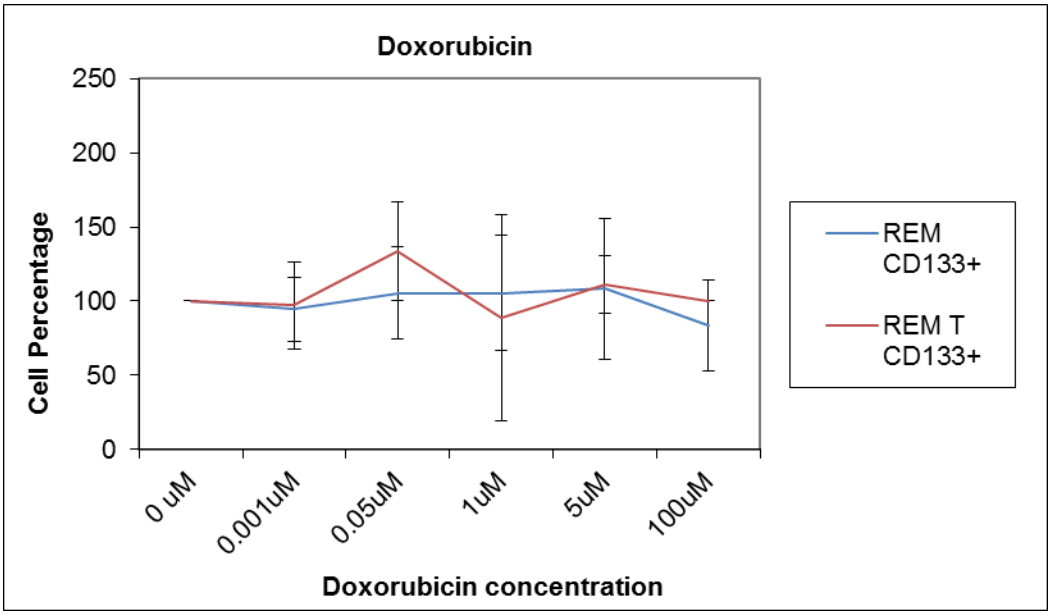

Figure 4.11. TGF-β-stimulated and unstimulated REM CD133+ cells were resistant to doxorubicin. REM cells expressing the stem cell marker CD133, treated with 10 ng/ml of TGF-β demonstrated no change in sensitivity to doxorubicin compared to vehicle-treated control CD133+ cells. REM CD133+ = CD133+ REM cells; REM T CD133+ = CD133+, TGF-β-stimulated REM cells. A Mann-Whitney test was carried out where the overall p-value was 0.7347 for the whole experiment.

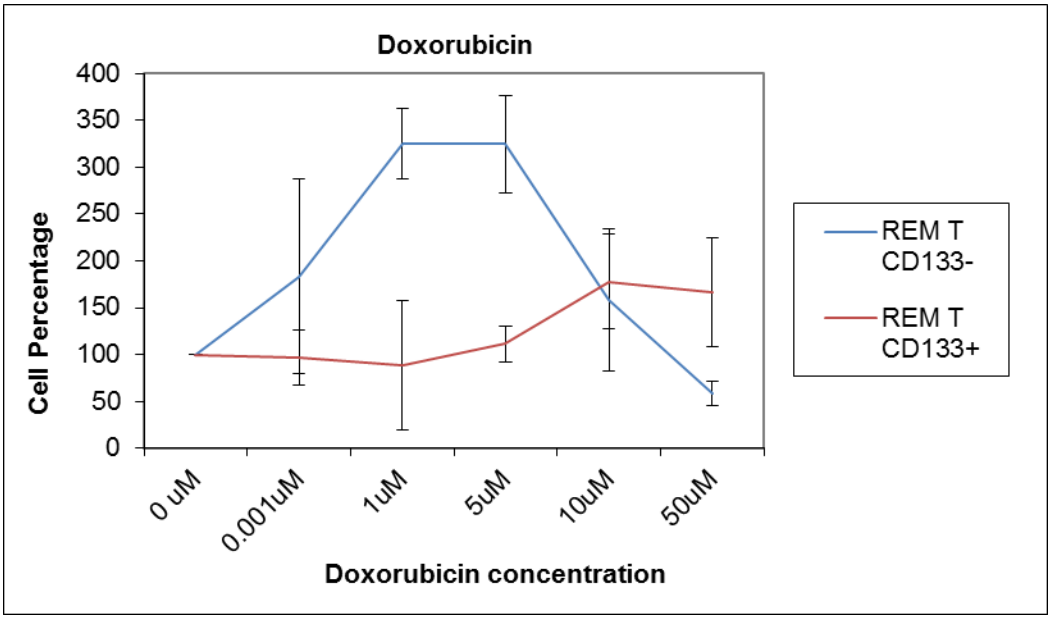

Figure 4.12. TGF-β-stimulated REM cells showed resistance to doxorubicin with and without expression of stem cell surface marker CD133. REM cells stimulated with 10 ng/ml of TGF-β showed resistance to the cytotoxic effects of doxorubicin, regardless of their expression of the stem cell marker CD133. REM T CD133- = CD133-, TGF-β-stimulated REM cells; REM T CD133+ = CD133+, TGF-β-stimulated REM cells. A Mann-Whitney test was carried out where the overall p-value was 0.08727 for the whole experiment. An ANOVA test was carried out for the comparison between the CD133+ and CD133- fractions at individual concentrations where p-values were 0.2432 for 0.001 µM of TGF-β, 0.00027 for 1µM, 0.0007618 for 5µM, 1 for 10µM, and 0.42078 for 50µM.

4.4 Discussion

Cancer cells with stem cell-like characteristics are more resistant to apoptosis and different therapeutic approaches including chemotherapeutic agents (Wright et al., 2008, Aomatsu et al., 2012). The mechanisms through which these cancer cells acquire tumorigenic and resistant traits have not been fully elucidated, nevertheless, cancer research is moving rapidly towards the understanding of these events.

Different research groups have confirmed the presence of tumorigenic properties in oncogenic cell lines of breast cancer in humans assessed by their sphere forming abilities under limiting survival conditions and the expression of stem cell surface markers including CD44+CD24- (Morel et al., 2008) and CD133 (Xiao et al., 2008). More importantly, they found that non-tumorigenic cell lines such as MCF10 after undergoing EMT expressed surface markers characteristic of stem cells suggesting that there could be a link between EMT and the acquisition of stem cell characteristics in human breast cancer cells (Morel et al., 2008). Similarly, *Chen et al* demonstrated that head and neck cancer cells expressing CD133 have stem cell-like characteristics, and show EMT-associated changes (Chen et al., 2011). To date similar features of breast cancer in companion animals have not been investigated. It is crucial to carry out studies to assess whether such companion animal species are able to generate breast cancer cells with stem cell-like characteristics and therefore if they can serve as useful animal models for the development of novel strategies to understand and combat the disease in humans.

Here we confirmed the presence of canine and feline breast CSCs, and that these cells have a more resistant phenotype than differentiated breast cancer cells in these species. These subpopulations of resistant cells have self-renewal properties demonstrated through their sphere forming ability while grown in limiting conditions. Spheres are considered putative CSCs as they self-renew and they are resistant to limiting conditions. Interestingly, we confirmed that cells previously treated with 10 ng/ml of TGF-β displayed morphological changes consistent with EMT in adherent culture, as shown in Chapter 3.3.1, and an enhanced sphere forming ability with changes in protein expression profile in both culture conditions

consistent with EMT. These findings are similar to those reported by other research groups with human cell lines (Morel et al., 2008, Lim et al., 2013). Interestingly, the canine and feline mammary carcinoma cell lines, REM and CMC, respectively showed downregulation of epithelial markers and upregulation of mesenchymal markers after forming spheres. With these findings we can confirm some pathophysiological similarities with which human and companion animal breast cancer cells acquire stem cell-like characteristics in association with an induced EMT. Similarly, *Mani et al., 2008* showed that exposure of human mammary epithelial and breast cancer cells to EMT inducers, including TGF-β, twist and snail, resulted in at least 40 fold more mammospheres than unstimulated control cells, with attendant increases in vimentin and fibronectin mRNA, and decreased E-cadherin expression. They concluded that the acquisition of sphere forming ability and the expression of these EMT-associated transcriptional changes can be attributed to cells acquiring stem cell characteristics after an induced EMT, similar to our findings in Chapter 4.3.1 (Mani et al., 2008).

Previous work in our lab showed that spheres harvested from canine mammary carcinoma cells without TGF-β stimulation resembled CSCs, acquired resistance to doxorubicin, became more invasive and expressed embryonic stem cell markers Oct4 and Nanog compared to adherent cells (Pang et al., 2011). Both the latter markers confer self-renewal characteristics and maintain the undifferentiated state of cells as reviewed by *Pan and Thomson, 2007*. Furthermore, in order to expand this previous study in our lab, Dr. Lisa Pang examined whether spheres have a mesenchymal phenotype compared to parental adherent cells at the protein expression level by western blot analysis. She showed that non-TGF-β-stimulated spheres displayed an evident decrease in the expression of epithelial markers (E-cadherin and β-catenin) whilst the expression of mesenchymal markers (fibronectin and vimentin) was increased, compared to parental adherent cells (Pang et al., 2011). Moreover, in keeping with EMT being associated with migration and invasion. The spheres exhibited a greater invasive potential than adherent cells (Pang et al., 2011). These observations confirmed that EMT and the CSC theory are closely related. We can conclude from this that CSCs can have invasive features, and EMT might be an

important promoter of the acquisition of CSC features, as spheres, which are considered putative CSCs, showed EMT-associated changes. These findings are in line with our results where we show that tumourspheres derived from canine and feline mammary carcinoma cells express several EMT-associated changes.

Xu et al., 2014 showed that the acquisition of stem cell-like characteristics associated with an induced EMT is also seen in non-small cell lung cancer (NSCLC) cells fused with bone marrow-derived mesenchymal stem cells. These hybrid cells were highly malignant and showed EMT-associated changes including a more mesenchymal morphology, upregulation of mesenchymal markers, downregulation of E-cadherin and acquired stem cell characteristics. These hybrid cells were capable of forming tumourspheres and showed overexpression of CD133, OCT4 and Nanog among other embryonic stem cell markers, similar to the data presented herein generated in our laboratory by Dr. Lisa Pang (Pang et al., 2011). It would be interesting to compare TGF-β exposure with fusion with bone marrow-derived mesenchymal stem cells in terms of CSC and EMT induction in veterinary breast cancer cell lines.

Furthermore, in order to confirm that these EMT-induced cells express stem cell surface markers, we submitted them to a MACS analysis and found that the EMT-induced cells had a larger CD133+ subpopulation than the untreated cells. These TGF-β-stimulated cells were also confirmed to have undergone an EMT by morphological changes and changes in protein expression prior to the cell sorting. In line with the observations described above by *Morel et al* and *Xiao et al,* these EMT-induced cells are capable of forming spheres and express stem cell markers, supporting a link between EMT and the acquisition of stem cell-like characteristics in canine and feline breast cancer cell lines.

Moreover, typically, cells with stem cell characteristics within a tumour comprise a very low percentage of the whole population (reviewed by Reya et al., 2001 and All Hajj et al., 2003), thus, the CD133+ cell subpopulations in our results were expected to be far smaller than the CD133- fractions. In line with this, we showed that the CD133 stem cell marker was expressed in only 3% and 1.3% of

mammary carcinoma cells of dogs and cats, respectively, after exposure to TGF-β. TGF-β exposure tripled and doubled the percentage of CD133+ cells within mammary carcinoma cells without TGF-β stimulation of dogs and cats, respectively, as expected. A study carried out by *Giordano et al*. in 2012 with HER2+ human mammary carcinoma-bearing patients showed a positive association between the expression of EMT-associated transcription factors (snail and ZEB1) and the percentage of CD133+ cells (Giordano et al., 2012). It would be interesting to submit our CD133+ and CD133- cells for EMT-associated marker protein and mRNA expression analyses to evaluate the similarities between humans and veterinary species in this respect. On a distinct approach, *Quintana et al* demonstrated that in human melanoma, by modifying xenotransplantation assays in immunodeficient mice, 1 in 9 melanoma cells were capable of forming tumours (Quintana et al., 2008). Interestingly, after individualisation of melanoma cells by flow cytometry, they observed that 27% of single cell injections developed tumours in immunodeficient mice. They used NOD/SCID mice lacking the interleukin-2 gamma receptor to confirm these findings. Their observations suggest that there might be a much higher frequency of human melanoma cells with tumorigenic traits than the reported frequency for other kinds of cancer (Quintana et al., 2008).

After observing that canine and feline mammary carcinoma cells became more resistant to limiting survival conditions and acquired enhanced sphere forming ability after a TGF-β-induced EMT, we assessed whether putative stem cells derived from TGF-β-stimulated cells also acquired resistance to chemotherapeutic agents. As previously stated in Chapter 4.2, *Nadal et al* observed that CD133+ breast cancer human patients had higher probabilities of tumour recurrence due to resistance to apoptosis and chemotherapy (Nadal et al., 2013). To confirm this, we subjected canine and feline mammary carcinoma cell lines with and without TGF-β stimulation to chemosensitivity assays with doxorubicin and mitoxantrone. It is known that cells with a faster growth rate are more susceptible to chemotherapy (reviewed by Argyle, 2008), but it has been thought that cells with resistant properties enter quiescent periods during chemotherapeutic protocols through an autophagy process, as reviewed by *Kimmelman, 2011*. This latency enables them to survive and eventually

cause relapse. In this study, EMT-induced cells and the CD133+ fractions of each cell line were expected to show greater resistance to chemotherapeutic drugs than the untreated cells and their CD133- subpopulations. Interestingly, the feline mammary carcinoma cell line, despite showing enhanced sphere-forming ability after TGF-β stimulation, did not show a change in resistance to doxorubicin or mitoxantrone after TGF-β stimulation. Interestingly, these EMT-induced cells contained a greater subpopulation of CD133+ cells compared to the untreated controls indicating that TGF-β stimulation does increase the percentage of putative CSCs in CMC, which does not translate into increased resistance to doxorubicin. A single feline cell line was used here and further experimentation on a panel of feline cancer cell lines is required to determine if this result is cell line or species-specific.

In contrast to our findings in CMC cells, TGF-β-stimulated REM cells showed a better survival rate than untreated cells when exposed to doxorubicin but not mitoxantrone. Both drugs are considered antineoplastic antibiotics and inhibit DNA and RNA synthesis, mitoxantrone being a synthetic form of doxorubicin. The main mechanism of action of both drugs is not fully understood and thus, it is difficult to find an explanation of why canine breast cancer cells are more resistant to doxorubicin than to mitoxantrone after an EMT induction.

Our results show that the TGF-β-stimulated REM cells survived better at all concentrations of doxorubicin compared to vehicle-treated, control cells. Furthermore, we decided to carry out more chemosensitivity assays with the REM cells to assess if there was a difference between TGF-β-stimulated and unstimulated cells and their respective CD133+ and CD133- fractions. Interestingly, CD133+ cells sorted from the untreated population survived doxorubicin exposure better than CD133- cells. This result shows that CD133+ cells, which might represent the CSC population in a tumour, are resistant to doxorubicin compared to the CD133-fraction from the same cell line. The similarity between these assays suggests a link between EMT and the acquisition of stem cell-like characteristics in canine mammary carcinoma cells. A study carried out by *Zhu et al*. in 2014 showed that in humans, CD133+ gastric carcinoma cells were more resistant to 5-fluorouracil, which they consider an important chemotherapeutic agent for advanced gastric cancer. Similar to

our findings, they confirmed that CD133+ cells were more resistant to the afore mentioned agent than CD133- cells. Furthermore, they silenced CD133 in gastric cancer cells with small interfering RNA against CD133 and observed that this enhanced the cytotoxicity effects conferred by the same agent (Zhu et al., 2014). The similarities between these findings and our chemosensitivity assays give an important idea for future plans in our search for more answers regarding chemoresistance in stem cell-like mammary carcinoma cells in dogs, cats and humans, and their association with EMT-induced stemness.

Moreover, in order to assess if CD133+ cell fractions from TGF-β-stimulated and unstimulated canine mammary carcinoma cells shared resistant characteristics, we subjected both to chemosensitivity assays and both proved equally resistant to doxorubicin. These results are consistent with CD133+ cells being representative of CSCs. To determine if the expression of CD133 is essential for doxorubicin resistance in EMT-induced cells, we compared TGF-β-stimulated CD133+ cells to TGF-β-stimulated CD133- cells as shown in Figure 4.12. Interestingly, both cell populations showed greater survival than non-TGF-β-stimulated, CD133- cells as shown in Figures 4.12 and 4.10, respectively. The CD133+ fraction showed a steady percentage of cell survival throughout the entire assay. The CD133- fraction showed resistance to doxorubicin at all but the highest concentrations used. It is of importance to mention that the data acquired from the TGF-β-stimulated REM cells and the TGF-β-stimulated CD133- REM cells look very similar in our assays, as shown in Figures 4.9 and 4.12, respectively. A possible explanation for this outcome would be that the percentage of the CD133+ cells was very low compared to the CD133- cells as reported in Chapter 4.3.2. The percentage of the CD133- cells within the TGF-β-stimulated REM cells was 97%, thus, the results for their chemosensitivity assays would be expected to be quite similar to sorted CD133- TGF-β-exposed REM cells.

This chemosensitivity assay proved that the expression of CD133, in this cell line, is not a necessary characteristic in EMT-induced cells to acquire resistance to doxorubicin, thus, probably there is a subpopulation of TGF-β-stimulated cells resistant to doxorubicin without expressing CD133 perhaps with other stem cell-like

properties. *Diehn et al., 2006* analysed research studies carried out by *Phillips et al*., 2006 regarding CSC markers and stem cell-like characteristics in breast cancer cells where they confirmed that CD24-/CD44+ cells were radioresistant (Phillips et al., 2006), and after an exhaustive inspection of this study, *Diehn et al., 2006* concluded that the expression or absence of stem cell markers does not always confirm the stem cell functional phenotype, as cancer cells with tumorigenic traits might not necessarily share specific cell surface markers even within the same cell line *in vitro* or *in vivo*. It has also been reported that in order to confirm that cells acquire stem cell-like properties, it is essential that these cells exhibit said characteristics, including self-renewal and resistant traits rather than just the expression of cell surface markers (reviewed by Diehn and Clarke, 2006).

Our data, showing the functional impact of TGF-β-induced EMT and CD133 expression suggest further investigation of EMT as a potential therapeutic target in companion animal oncology. The TGF-β pathway including its transcription factors could be targeted to combat breast cancer progression and metastasis. The understanding of how TGF-β might be involved in the induction and/or plasticity of CSCs is of great importance to achieve meaningful insights for future TGF-β-directed cancer therapeutics in human and veterinary oncology. Further investigation is needed to confirm these findings *in vivo* as is the assessment of the impact of TGF-β-directed therapies on vital functions such as wound healing and tissue remodelling during cancer treatment.

Chapter 5: Global changes in microRNA levels during Epithelial to Mesenchymal Transition in dogs

5.1 Abstract

MicroRNAs (miRNAs) are small RNA molecules involved in a variety of vital processes. They are also involved in many diseases, including cancer. They can promote or regulate cancer-related genes, but they can also be targeted by different means such as different mRNAs or other miRNAs. The role of miRNAs in EMT has yet to be fully elucidated. Previous studies have shown a role for several miRNAs, including the miRNA 200 family in metastasis of breast cancer, lung carcinoma and cervical cancer in humans (Iorio et al., 2005, Cheng et al., 2005). Moreover, since 2008, different research groups have been correlating the association of specific miRNA expression in different types of cancer in humans and companion animals, including mammary carcinoma and lymphoma (Boggs et al., 2008, Uhl et al., 2011, von Deetzen et al., 2014 and Mohammad et al., 2015). In this study we aimed to determine changes in miRNA expression through a thorough screening of canine mammary carcinoma cells during TGF-β-induced EMT. We found significant changes regarding the expression of different miRNAs in TGF-β-stimulated canine mammary carcinoma cells compared to untreated cells. Future research on the role of miRNAs in cancer could lead to a better understanding of how they are involved in tumour progression and metastasis.

5.2 Introduction

MicroRNAs (miRNAs) are small RNA molecules with 19-25 nucleotides (reviewed by Ambros, 2004, Hwang and Mendell, 2006) which can be associated with several organic functions including fundamental physiological cell processes such as tissue differentiation, but they can also be involved in the development and progression of different diseases like cancer (Foubert et al., 2010). They work in a post-transcriptional fashion, guiding the RNA-induced silencing complex and targeting their complementary mRNAs which are then translationally silenced or cleaved (reviewed by Bartel, 2004 and Hwang and Mendell, 2006). One miRNA can target several mRNAs and can also be targeted by several mRNAs (reviewed by Bartel, 2004) as detailed in Chapter 1.15. The understanding of different processes through which miRNAs act is essential to elucidate how these small RNA molecules repress or promote tumour suppressor genes in order to develop novel therapeutics against specific types of cancer as reviewed by *Esquela-Kerscher and Slack, 2006*.

Research studies have shown a correlation between different families of miRNAs and oncogenesis in human cancers. Deregulation of miR-143, miR145 and miR-125 among other miRNAs has been correlated to cancer arising in different human tissues including colorectal, breast, prostate, cervical, lymphoid and pancreatic tissues (Michael et al., 2003, Kent et al., 2010, Feliciano et al., 2013, Yin et al., 2014 and Zhang et al., 2014). *Michael et al., 2003* showed that in cancer cells from human breast, prostate, cervical, colorectal and lymphoid cancers the expression of miR-143 and miR-145 is downregulated (Michael et al., 2003). On a similar basis, *Kent et al., 2010* also found significant reduced expression of miR-143 and miR-145 in mice xenograft models of human pancreatic adenocarcinoma cells harbouring KRAS mutations when compared with normal pancreatic tissue (Kent et al., 2010). Furthermore, *Yin et al., 2014* showed that the expression of miR-145 is strongly downregulated in plasma and colorectal tissues of human patients with colorectal cancer (Yin et al., 2014). Regarding the expression of miR-125 in human cancer, Feliciano et al., 2013 demonstrated that amongst 939 analysed miRNAs, miR-125b showed the most profound decrease in expression in breast cancer. They analysed breast cancer tissues and adjacent normal breast tissues from 50 breast

cancer patients and concluded that miR-125b acts as a tumour suppressor gene in breast cancer in humans (Feliciano et al., 2013).

Consistently reduced levels of miRNA expression in cancer compared to normal tissues indicate their possible direct involvement in carcinogenesis and tumour progression as shown by the afore mentioned research groups (Michael et al., 2003, Kent et al., 2010, Feliciano et al., 2013 and Yin et al., 2014). Interestingly, opposed to the observations of *Feliciano et al., 2013* in breast cancer patients, *Zhang et al., 2014* showed that miR-125b is strongly expressed in squamous cell carcinoma of humans and mice at early stages of malignant progression and also promotes tumour initiation in mice (Zhang et al., 2014). These opposing observations made by *Feliciano et al., 2013* and *Zhang et al., 2014* regarding the expression of miR-125b in different types of cancer might give insights on how the expression of specific miRNAs may be cancer type-dependent.

Iorio et al, 2005 identified a set of 15 miRNAs the pattern of expression of which could accurately define whether human breast tissues were normal or cancerous, due to a clear difference in miRNA expression between breast cancer and normal tissues. They found consistent deregulations in miR-145, miR-10b, miR-125b, miR-155 and miR-21 confirmed by northern blot analysis and microarray. (Iorio et al., 2005). Among these miRNAs, miR-21 and miR-155 were upregulated whilst miR-10b, miR-125b and miR-145 were downregulated in breast cancer tissue, compared with normal breast tissue, suggesting their potential activity as oncogenes and tumour suppressor genes, respectively. They also identified possible targets of these deregulated miRNAs. Tumour suppressor genes are possible targets of the upregulated miRNAs and oncogenes possible targets of the downregulated miRNAs. They used three different algorithms including miRansa, TargetScan and PicTar as methods to detect human miRNA target genes and they found that TGF-β is a predicted target of miR-21. Mir-21 was progressively upregulated in breast cancer with higher tumour stages. In contrast, miR-145 was found to be downregulated in breast cancer tissues and negatively correlated with high proliferation index. These data imply that changes in miRNA expression might be associated, perhaps causally, with molecular events at specific stages of cancer progression (Iorio et al., 2005).

Screening for miRNA expression profiles is a useful tool to investigate their involvement in cancer and its progression. *Cheng et al., 2005* identified miRNAs that influenced *in vitro* cell growth regulation and apoptosis in different experiments in both a cervical cancer-derived cell line (HeLa) and a lung carcinoma cell line (A549). They found that individual inhibition of 19 different miRNAs caused a decreased cell growth rate, but that inhibition of miR-21 and miR-24 caused an increase in cell growth in HeLa cells. MiR-21 is suggested to be a cell growth regulator in HeLa cells *in vitro*. These findings are opposed to the observations of *Iorio et al., 2005* observed where miR-21 expression was upregulated in breast cancer tissue. When compared to A549 cells, *Cheng et al.,* 2005 found 3 miRNAs inhibition of which caused decreased cell growth, but inhibition of the miRNAs under study was not associated with increased growth *in vitro*. Moreover, they found that inhibiting some miRNAs produced opposing effects on growth rate in the cell lines under study. For example, an increased cell growth in HeLa cells but a decrease in that of A549 cells after miR-24 inhibition was observed. Similarly, inhibition of miR-191 caused an increase in the cell growth rate of A549 cells but a decrease in that of HeLa cells. These three examples including miR-21, miR-24 and miR-191 and their differences in these cell lines, suggest that miRNAs might have cell type specific effects: different targets for each cell line; or perhaps miRNAs are differentially expressed in different cell lines. The same research group also screened miRNAs to assess their involvement in apoptosis in HeLa cells. They observed that the inhibition of 8 different miRNAs including 4 from the miR-200 family increased the level of apoptosis measured through caspase activity assays (Cheng et al., 2005). The variation in the effects of miR-21, miR-24 and miR-191 strongly suggests that miRNAs have cell and context specific effects.

MicroRNA expression has also been studied in companion animals by several research groups. Researchers have been correlating the association of specific miRNA expression with different types of cancer in humans and companion animals, including lymphoma and mammary carcinoma (Boggs et al., 2008, Uhl et al., 2011, von Deetzen et al., 2014 and Mohammad et al., 2015). *Boggs et al., 2008* investigated the role of specific miRNAs in canine mammary carcinoma and

observed similar results to those from human research (Boggs et al., 2008). They analysed the expression patterns of 10 specific miRNAs (miR-15a, miR-16, miR-17-5p, miR-21, miR-29b, miR-125b, miR-145, miR-155, miR-181b and let-7f) in canine mammary tumours and normal canine mammary tissues, as these specific miRNAs are known to be associated with human mammary malignancies (reviewed by Calin et al., 2004 and Boggs et al., 2008). Interestingly, they observed that from all analysed canine miRNAs, only miR-145 did not follow a similar pattern of expression as in human samples (Boggs et al., 2008). *Von Deetzen et al., 2014* extended these findings by comparing the expression of 16 specific miRNAs not only between canine mammary cancer and normal gland samples, but also between samples of tumours with different malignancies, including adenomas, metastasising and non-metastasising carcinomas and lymph node metastases. They found that the expression of 5 miRNAs (miR-29b, miR-101, miR-125a, miR-143 and miR-145) was different between primary tumours and their metastases. When they compared primary tumours of different aggressiveness, they only identified one significant difference in miRNA expression, with miR-125a expression being significantly different between metastasising carcinoma and adenoma (Von Deetzen et al., 2014). Based on these observations, further investigation is needed to assess the differences in miRNA expression between canine mammary tumours at different stages in order to elucidate potential therapeutic targets.

In 2011 a different research group observed that the expression of specific miRNAs associated with apoptosis, differentiation and cancer in human lymphoma was similar to that of naturally occurring canine B- and T-cell lymphomas (Uhl et al., 2011). They used a human cancer miRNA qPCR array to assess miRNA expression in canine lymphoid cell lines and naturally occurring lymphomas compared with normal canine peripheral blood mononuclear cells and healthy lymph nodes. Upregulation in the expression of miR-19-apb and miR-17-5p, and a decreased expression of miR-203, miR-218 and miR-181a was observed (Uhl et al., 2011), consistent with miRNA expression in human lymphoid malignancies as reviewed by *Khana et al., 2006*. Based on these observations, *Uhl et al., 2011* emphasise that

spontaneous canine lymphoma may act as a useful animal model of human lymphoma.

MicroRNA activity has also been inhibited efficiently *in vivo* by *Krutzfeldt et al., 2005* utilising antimiRNA oligonucleotides (AMOs) against human miR-16, miR-122, miR-192 and mir-194 injected intravenously in mice. They utilised a cholesterol conjugation in order to improve delivery of these miRNA inhibitors. AMOs are small interfering double-stranded RNAs (siRNAs) engineered as cholesterol-conjugated single-stranded RNA analogues which are complementary to the targeted miRNAs. The inhibition of these miRNAs resulted in a remarkable reduction in miRNA levels in lungs, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenal glands. These chemically engineered oligonucleotides were called antagomirs (Krutzfeld et al., 2005). These findings suggest that antagomirs may become a potential therapeutic approach for silencing specific miRNAs in several diseases including different types of cancer.

In the case of miRNAs acting as tumour suppressor genes or miRNAs inhibiting oncogenes, delivery techniques including viral and liposomal transfection have been studied in order to more accurately elucidate miRNA roles in cancer and potentially develop therapeutic protocols, as reviewed by *Izquierdo, 2005* and *Zhang et al., 2013*. These delivery techniques could be utilised for the treatment of specific types of tumours, as observed by different research groups (Zhu et al., 2008, Takeshita et al., 2010 and Wu et al., 2013). *Zhu et al., 2008* were able to suppress miR-21 in human metastatic breast cancer cells after successful delivery of antisense miR-21 oligonucleotide (anti-mir-21). They observed a significant reduction in cell invasion after cell transfection *in vitro*, whilst the number of lung metastases was lower in young female nude mice transfected with anti-miR-21 *in vivo*, compared with mice bearing negative control-transfected cells. They also assessed the inhibition of invasiveness in metastatic prostate cancer cells after treatment with anti-miR-21 and found similar results (Zhu et al., 2008). On a similar approach, *Takeshita et al., 2010* transfected human prostatic carcinoma cells with synthetic miR-16 and observed a significant reduction in cell proliferation *in vitro*. Moreover, they successfully delivered synthetic miR-16 to metastatic prostatic tumours in bone

tissues in mice after injection into their tail veins and observed significant growth inhibition in these tumours *in vivo* (Takeshita et al., 2010),thus, indicating that systemic delivery of miRNAs could be used to treat cancer patients. Furthermore, *Wu et al., 2013* developed a cationic lipoplexes-based carrier to efficiently deliver miR-29b to non-small-cell lung carcinoma cells and showed that cell growth was strongly reduced *in vitro* after delivery of miR-29b. Also, after miR-29b systemic transfection into a xenograft murine model *in vivo*, tumour miR-29b expression was increased by approximately fivefold and tumour growth was inhibited by approximately 60% compared with negative control-transfected mice. They concluded that their delivery system could be considered an important potential tool for the development of miRNA-based therapeutics for lung cancer (Wu et al., 2013).

Optimisation of delivery of miRNAs to reach pharmacologic targets is the key to robustly assess the effectiveness of these approaches *in vivo*, as reviewed by *Zhang et al., 2013*. This research group emphasises the importance of further studying miRNA delivery techniques as to diminish the concerns about cells evading treatment, especially in diseases like cancer, for which conventional medicine has proven ineffective (reviewed by Zhang et al., 2013). It has been observed that the efficacy of cancer gene therapies can be limited by the immune system response of the organism (Gitlin et al., 2002 and Sledz et al., 2003, reviewed by Zhang et al., 2013).

Future research in this field will provide important information regarding the role of miRNAs in different stages of cancer progression and oncogenesis, and might give rise to novel therapeutic targets for specific tumours and their metastases (Zhu et al., 2008, Takeshita et al., 2010, Wu et al., 2013 and reviewed by Esquela-Kerscher and Slack, 2006 and Zhang et al., 2013). The future development of novel techniques to inhibit or to transfect miRNAs will not only be useful to inactivate or to overexpress known miRNAs in specific tumours as therapeutic options, but could also elucidate their involvement in different stages of cancer progression and in manipulable model systems.

In this study, we mapped global changes in miRNA expression during TGF-β-induced EMT in canine mammary carcinoma cells at different time points. We observed significant changes in miRNA expression over time. We observed that 5 different miRNAs were upregulated in TGF-β-stimulated canine mammary carcinoma cells over time compared to cells treated only with vehicle. Interestingly from these 5 upregulated miRNAs, cfa-miR-411, 381, 410 and 380 showed peak expression by day 10 of stimulation and started decreasing their expression thereafter. The remaining miRNA (cfa-miR-363) showed an overexpression until day 23 of TGF-β treatment. Previous studies in human cancer have determined that these 5 miRNAs have roles in carcinogenesis: miR-380 and miR-363 are overexpressed in neuroblastoma and breast cancer, respectively (Swarbrick et al., 2010, Beltran et al., 2011); miR-411 and 381 are downregulated in breast cancer; and miR-410 is downregulated in endometrial carcinoma (van Schooneveld et al., 2012, Liu et al., 2013, Torres et al., 2013). Our findings provide insight into the regulation of EMT in canine cancer cells and identify several potential targets, which require further investigation.

5.3 Results

5.3.1 Target identification

Prior to screening canine mammary carcinoma cells for miRNA expression we needed to identify EMT-associated changes in TGF-β-stimulated cells to assess the correlation between EMT and miRNA expression in breast cancer in dogs. We identified EMT-associated changes in REM cells at 6, 12, 18 and 24 days after treatment with 10 ng/ml of TGF-β. Morphological changes were observed over 24 days. Untreated cells from day 6 and vehicle control-treated cells from day 6 were utilised throughout the experiment, as TGF-β-stimulated cells started showing mesenchymal traits at day 6 and kept these characteristics until day 24. We stimulated cells with 10 ng/ml of TGF-β for 24 days and extracted RNA as described in Chapter 2.6.1.2 at days 6, 12, 18 and 24. Previously we have observed that withdrawal of TGF-β caused a reversion of morphological changes, for which we sought to investigate whether there was an associated change in miRNA expression profile. TGF-β was withdrawn from stimulated cells after 19 days of treatment and interestingly, we found that cells started showing morphological changes consistent with an epithelial phenotype such as polygonal shape and increased cell to cell contact (data not shown). We also extracted RNA from these cells at day 24 (5 days after TGF-β withdrawal) in order to compare their miRNA expression profile with control and stimulated cells. These cells will be called TGF-β withdrawal cells.

We initially determined mRNA expression levels of EMT markers including E-cadherin, vimentin and fibronectin; and house-keeping gene RPL32, as described in Chapter 2.7.3. We found EMT-associated changes in mRNA expression from day 6 until day 24 with perhaps the most significant changes occurring at day 18. Downregulation of the epithelial marker E-cadherin and upregulation of mesenchymal markers vimentin and fibronectin are considered hallmarks of EMT (Bolos et al., 2003). We found that E-cadherin mRNA expression was slightly downregulated at day 6 and 12 of TGF-β stimulation, and further downregulated by day 18 and 24. In the TGF-β withdrawal sample, E-cadherin expression was slightly higher than in TGF-β-stimulated cells at day 18 and day 24 of TGF-β stimulation.

This finding would suggest that cells return to a more epithelial phenotype after EMT due to a withdrawal of TGF-β by day 19 of stimulation (Figure 5.1).

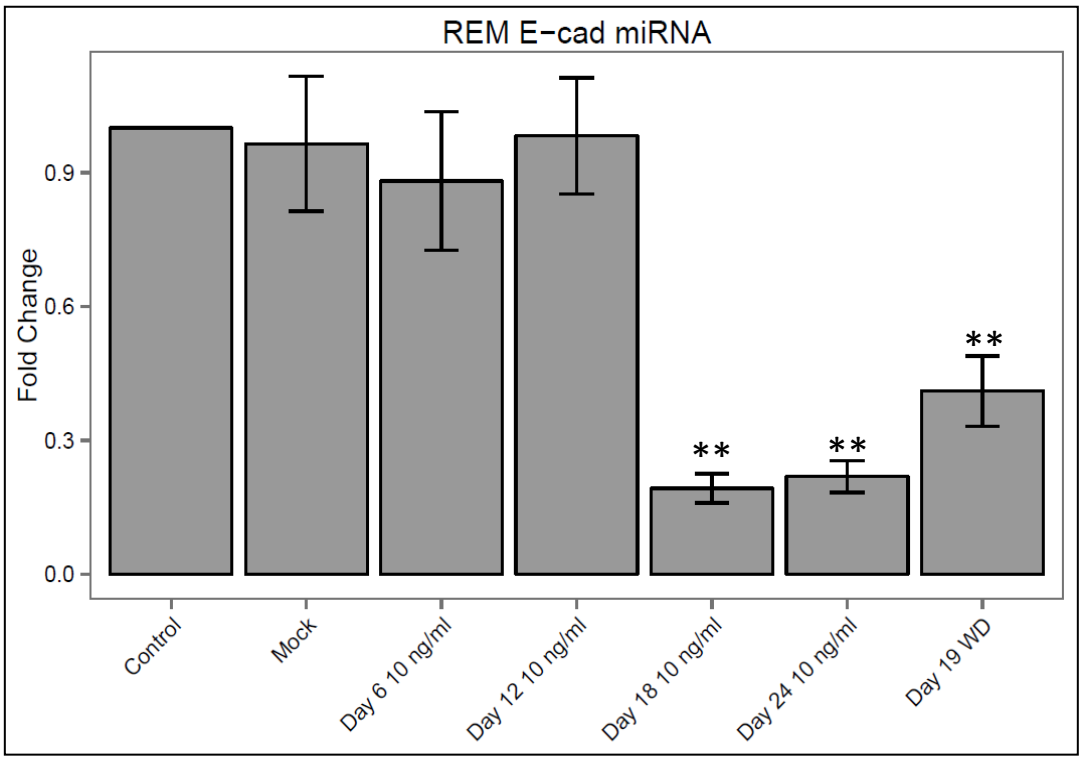

Figure 5.1 . Canine mammary carcinoma (REM) cells show a significant downregulation of the mRNA expression of the epithelial marker E-cadherin after 18 and 24 days of stimulation with 10 ng/ml of TGF-β. E-cadherin expression is slightly upregulated after TGF-β withdrawal compared to days 18 and 24 of treatment. Control = untreated cells from day 6, Mock = vehicle-treated cells for 6 days. Day 6 10 ng/ml = cells harvested at day 6 of TGF-β treatment, Day 12 10 ng/ml = cells harvested at day 12 of TGF-β treatment, Day 18 10 ng/ml = cells harvested at day 18 of TGF-β treatment, Day 24 10 ng/ml = cells harvested at day 24 of TGF-β treatment, Day 19 WD = cells harvested at day 24 after TGF-β withdrawal at day 19. P values comparing stimulated samples with control cells: ** = p value ≤ 0.005

In contrast to E-cadherin, mRNA expression of the mesenchymal marker vimentin was significantly upregulated by days 6 and 12 and also upregulated at day 24 compared to the control and mock-treated cells as shown in Figure 5.2. It is important to mention that these EMT associated markers do not necessarily show changes at the same time as they are different genes with different functions during EMT as described in Chapter 1.9. Interestingly, the level of mRNA expression of vimentin in the TGF-β withdrawal samples was intermediate between that of day 18 of TGF-β stimulation and earlier time points, consistent with a partial reversal of the EMT-induced mRNA expression changes.

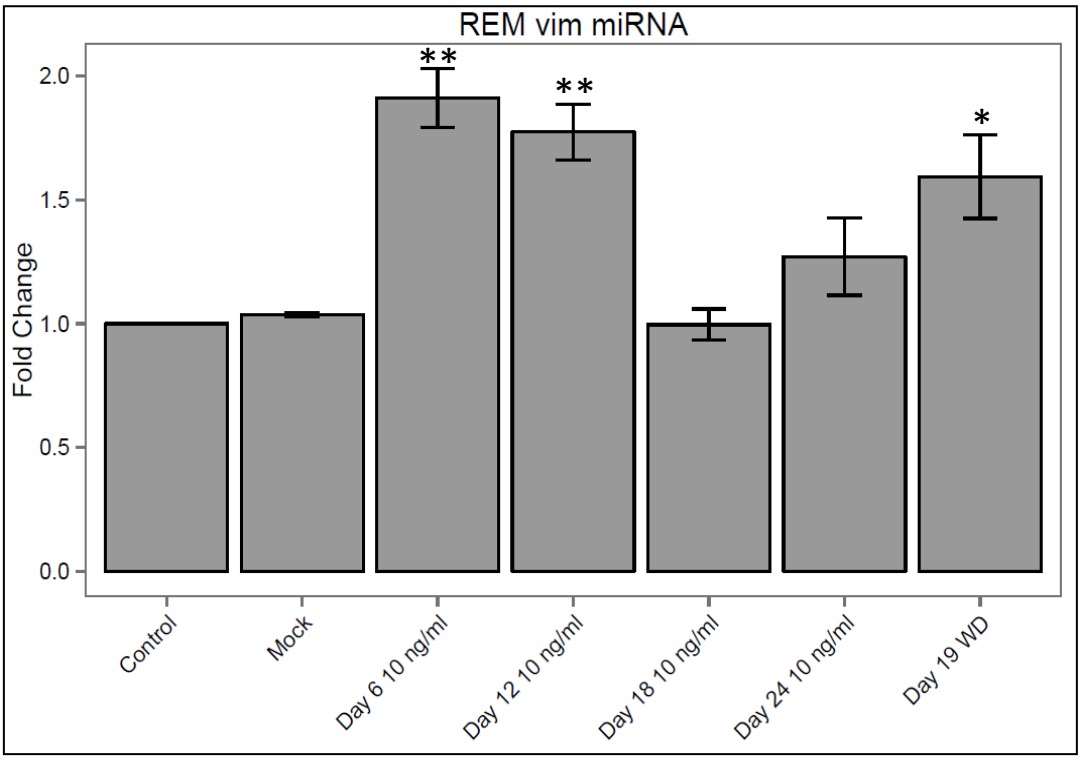

Figure 5.2. REM cells significantly upregulate the expression of mRNA of the mesenchymal marker vimentin after 6 and 12 days of TGF-β-induced EMT. Control = untreated cells from day 6, Mock = vehicle treated cells for 6 days. Day 6 10 ng/ml = cells harvested at day 6 of TGF-β treatment, Day 12 10 ng/ml = cells harvested at day 12 of TGF-β treatment, Day 18 10 ng/ml = cells harvested at day 18 of TGF-β treatment, Day 24 10 ng/ml = cells harvested at day 24 of TGF-β treatment, Day 19 WD = cells harvested at day 24 after TGF-β withdrawal at day 19. P values comparing stimulated samples with control cells: * = p value ≤ 0.05. ** = p value ≤ 0.005

Expression of mRNA of the mesenchymal marker fibronectin was markedly increased with TGF-β stimulation at all time points compared to untreated and mock-treated cells. REM cells stimulated with TGF-β for 6, 12 and 18 days showed a progressive upregulation of fibronectin mRNA. By day 24, fibronectin mRNA was also significantly upregulated compared to control and mock-treated cells, but less profoundly than at day 18 of TGF-β stimulation as shown in Figure 5.3. Similarly to our findings regarding mRNA expression of vimentin described in Figure 5.2, the level of mRNA expression of fibronectin in the TGF-β withdrawal samples was lower than that of day 18 of TGF-β stimulation, but higher compared to earlier time points, also suggesting a partial reversal of the EMT-induced mRNA expression changes.

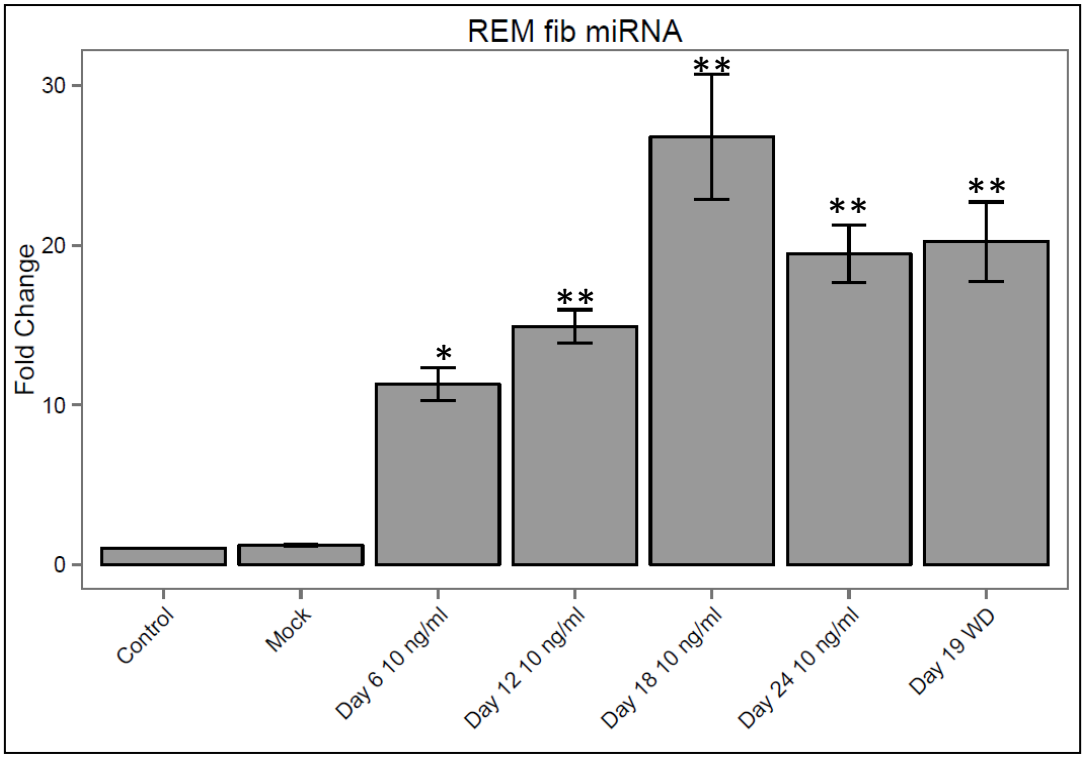

Figure 5.3. REM cells show significant upregulation of mRNA of the mesenchymal marker fibronectin after TGF-β stimulation throughout the entire assay. Control = untreated cells from day 6, Mock = vehicle-treated cells for 6 days. Day 6 10 ng/ml = cells harvested at day 6 of TGF-β treatment, Day 12 10 ng/ml = cells harvested at day 12 of TGF-β treatment, Day 18 10 ng/ml = cells harvested at day 18 of TGF-β treatment, Day 24 10 ng/ml = cells harvested at day 24 of TGF-β treatment, Day 19 WD = cells harvested at day 24 after TGF-β withdrawal at day 19. P values comparing stimulated samples with control cells: * = p value ≤ 0.05. ** = p value ≤ 0.005

5.3.2 MicroRNA expression analysis

After confirming EMT-associated mRNA expression changes by qRT-PCR during the TGF-β time course described above, REM cells were harvested and RNA extracted after stimulation with 10 ng/ml of TGF-β at day 1, 10, 17 and 23. We utilised samples harvested on these days as cells demonstrated a more mesenchymal phenotype from day 6 to day 24 confirmed by changes in morphology, translation, transcription, migration and invasiveness, as demonstrated in Chapters 3.3.1, 3.3.2, 3.3.3, 3.3.4 and 3.3.5, respectively. Moreover, cells treated with TGF-β for 19 days were harvested at day 23 (4 days after TGF-β withdrawal) in order to investigate if cells return to a more epithelial phenotype and exhibit associated changes in their miRNA expression after TGF-β withdrawal. Cells harvested on day 1 after TGF-β stimulation were chosen to be analyzed in order to map changes at the beginning of a TGF-β-induced EMT and to investigate whether epithelial breast cancer cells exhibit miRNA changes in advance of phenotypic changes during early TGF-β stimulation. Likewise, RNA was extracted with the same protocol from REM cells treated only with vehicle solution (mock-treated cells) which were harvested at the same time points as the treated cells.

All RNA extracts were sent to Edinburgh Genomics-Roslin Institute for miRNA sequencing as described in Chapter 2.12. In order to screen REM RNA samples for miRNA expression, all reads were mapped to known canine miRNAs. The reads left unmapped were then mapped to known human miRNAs. All counts were normalised to reads per million (RPM) mapped sequences and were organised where miRNAs were displayed in rows and the comparative experiments in columns in order to carry out a comparative study between the miRNA profiles of our samples as displayed in Appendix 2.

5.3.2.1 Experiment design

The differential expression analysis between samples was designed to compare miRNA expression in mock-treated cells and TGF-β-stimulated cells at each time point, and also to compare miRNA expression between both groups and the TGF-β withdrawal sample at day 23 as shown in Table 5.1.

Mock day 1	v	TGF-β day 1
Mock day 10	v	TGF-β day 10
Mock day 17	v	TGF-β day 17
Mock day 23	v	TGF-β day 23
Mock day 23	v	TGF-β withdrawal
TGF-β day 23	v	TGF-β withdrawal

Table 5.1 MicroRNA differential analysis in canine mammary carcinoma cells with and without TGF-β stimulation. MiRNA expression in mock-treated cells was compared to expression in contemporaneous TGF-β-stimulated cells. MicroRNA expression of TGF-β withdrawal samples was compared to both mock-treated and TGF-β-stimulated groups at day 23.

This type of analysis is concerned with differential expression rather than with the quantification of expression levels. It is concerned with relative changes in expression levels between conditions, but not directly with estimating absolute expression levels. Based on these concerns, all counts from mock-treated, TGF-β-stimulated and TGF-β withdrawal samples were normalised to reads per million (RPM) mapped sequences and were analysed for fold change (LogFC) and average counts per million (LogCPM). In order to evaluate the significance of the differences between miRNA expression groups, p-values incorporated false discovery rate (FDR) using the Benjamini and Hochberg correction as explained in Chapter 2.12. Due to the experimental design, there were not many miRNAs that passed the FDR≤ 0.05 filter, as would be expected when there are no biological replicates.

Positive and negative fold change (LogFC) values represent upregulated and downregulated expression, respectively. Average count per million (LogCPM) is a subjective continuous variable utilised to evaluate the relative level of expression, and as we do not have technical or biological replicates, these variables should be considered descriptive rather than absolute. High values showed that miRNAs are highly expressed and low values show that miRNAs are lowly expressed, regardless

if their fold changes were upregulated or downregulated. Whether any particular value for logCPM is high or low is subjective and should always be analysed through a comparative differential expression analysis rather than as an absolute count for one condition alone.

5.3.3 Multidimensional scaling analysis

All samples were analysed in a multidimensional scaling (MDS) analysis after miRNA mapping to compare similarities between samples, as described in Chapter 2.12. This model was constructed to primarily produce a simple and easily assimilated geometrical picture of our data, where distances between each point represent the empirical dissimilarities between our samples. These points are placed in such a way that the numerical value of their correlation coefficients is reproduced by the distances between them. A large matrix of numbers would be considerably more difficult to visually assimilate than a picture of the data (Coxon et al., 1982a). By these means, it is practically impossible to interpret the whole data configuration, but we could aim to discover a structure within parts of our data.

We are comparing a set of 9 samples for which we needed to establish how dissimilar they are to each other. MicroRNA profiles of cell samples showed outstanding separations along the first dimension (x-axis), in which mock-treated cells and TGF-β-stimulated cells separate remarkably as two large groups, where the highest level of dissimilarity was represented between TGF-β-stimulated cells at day 10 and mock-treated cells at day 10. The TGF-β withdrawal sample miRNA profile is closer to that of the mock-treated cells than to that of the TGF-β-stimulated samples (Figure 5.4).

Interestingly, mock-treated and TGF-β-stimulated samples from day 1 are relatively close in both dimensions, meaning that after only 1 day of treatment, TGF-β-stimulated cells miRNA expression is not very different to that of mock-treated cells. Apparently, miRNA profiles of mock-treated samples from day 10 to day 23 are very similar to each other, compared to the miRNA profiles of TGF-β-stimulated cells at the same time points which appear more widely dispersed along dimension 1. Moreover, TGF-β-stimulated cells miRNA profiles from days 10, 17 and 23 are

remarkably separated from that of TGF-β-stimulated cells at day 1 along dimension 2 in contrast with mock-treated cells, in which miRNA profiles show a lower degree of dissimilarity to each other. This could perhaps be explained knowing that our previous results show that cells change their phenotype after TGF-β stimulation in a time- and dose-dependent manner, as described in Chapters 3 and 4.

Interestingly, the degree of dissimilarity of miRNA expression profiles along dimension 1 between TGF-β withdrawal cells and mock-treated cells is lower than that between TGF-β withdrawal and TGF-β-stimulated cells. However, along dimension 2, TGF-β withdrawal cells miRNA expression profile appears more similar to that of TGF-β stimulated cells. This result could suggest that after TGF-β withdrawal, cells might regain some epithelial features but their miRNA expression is different with respect to mock-treated cells and cells continuously treated with TGF-β, conceivably displaying an intermediate state or cellular plasticity.

As mentioned above, it would be difficult to analyse all the information contained in all the correlation coefficients (Coxon et al., 1982b), although some features are obvious. Mock-treated cells at day 17 and 23 are clearly very much alike, as are TGF-β-stimulated cells at the same days, as indicated by their low degree of dissimilarity (short distance between each point). In contrast, the dissimilarity between mock-treated cells and TGF-β-stimulated cells in general is remarkable along dimension 1. One of the main objectives of this MDS analysis is to discover unnoticed characteristics of the data as depicted in numerical values (Figure 5.4).

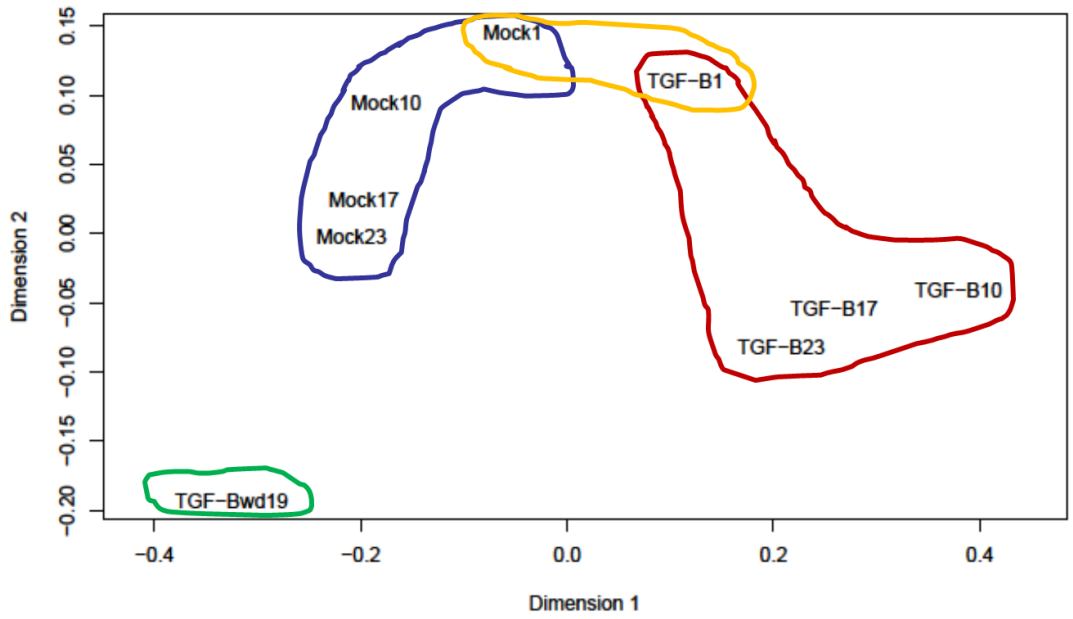

Figure 5.4. Multidimensional scaling analysis. Mock-treated cells (blue) separate from the TGF-β-stimulated cells (red) along the first dimension. Mock-treated and TGF-β-stimulated cells are least distinct in miRNA expression on day 1 of treatment (yellow). TGF-β withdrawal sample (green) appears closer to mock-treated cells than to TGF-β-stimulated cells along the first dimension, but appears remarkably separated from mock-treated cells. The TGF-β withdrawal sample is more similar to TGF-β-stimulated cells at days 10, 17 and 23 along the second dimension than to mock-treated cells. Mock 1, 10, 17, 23 = mock-treated cells harvested on days 1, 10, 17 and 23, respectively. TGF-B 1, 10, 17, 23 = TGF-β-stimulated cells harvested on days 1, 10, 17 and 23, respectively. TGF-Bwd19 = Cells from which TGF-β stimulation was withdrawn at day 19, harvested at day 23.

5.3.4 Mapping changes in miRNA expression during TGF-β-induced EMT in canine mammary carcinoma cells

In Figures 5.5, 5.6, 5.7 and 5.8, we show that 33 miRNAs from all miRNAs screened showed changes in expression as they were upregulated (positive logFC) or downregulated (negative logFC) in TGF-β-stimulated cells normalised to mock-treated cells for all different time points. A majority of miRNAs are upregulated, including at day 1 of TGF-β stimulation as shown in Figure 5.5. Moreover, at day 10 of TGF-β stimulation, only one miRNA was downregulated as shown in Figure 5.6. The results from days 17 and 23 of TGF-β stimulation show all miRNAs are

upregulated, as shown in Figure 5.7 and Figure 5.8, respectively. Also, when assessing miRNA expression in TGF-β withdrawal samples normalised to TGF-β-stimulated cells at day 23, only one miRNA was upregulated whilst the rest of them were downregulated as shown in Figure 5.9. Interestingly, miRNA expression analysis of TGF-β withdrawal samples normalised to mock-treated cells, showed that 4 of these 33 miRNAs were not expressed at all (cfa-miR-376b, cfa-miR-369, hsa-miR-1197 and cfa-miR-543). Also, the results of this particular study are ambiguous as 8 miRNAs were upregulated whilst 21 miRNAs were downregulated as shown in Figure 5.10. All these observations appear similar to what was observed in the MDS analysis results where the TGF-β withdrawal cells were different from both the mock-treated and the TGF-β-stimulated cells.

As previously explained, the MDS analysis in Chapter 5.3.3 should not be considered significant prior to a complete comparative study as we need to elucidate which properties are shared between our samples regarding miRNA expression. Even if we discovered the tendency of variation between our samples after the MDS analysis, and our comparative miRNA expression results are similar to the MDS data, it would be subjective to affirm that our comparative results are fully consistent with what we observed in the MDS analysis. Nevertheless, we can observe that the MDS analysis can suggest a general differentiation without specifying individual miRNA expression changes. For example, we could possibly correlate our comparative results at day 10 in Figure 5.6 with the MDS plot in Figure 5.4, where TGF-β-stimulated cells normalised to mock-treated cells show the greatest fold changes in upregulated miRNAs (Figure 5.6) consistent with the greatest separation between TGF-β-stimulated and mock-treated cells in dimension 1 (Figure 5.4). Also, comparing the logFC data generated from TGF-β withdrawal cells normalised to both mock-treated cells and TGF-β-stimulated cells at day 23 with the MDS analysis, in both miRNA expression is opposite in direction in TGF-β withdrawal cells when compared to TGF-β-stimulated cells and mock-treated cells, in dimension 1 of the MDS analysis plot. Moreover, changes in dimension 2 of the MDS analysis plot could also be correlated with our logFC data showing that the miRNA expression is in the same direction but more profound in TGF-β withdrawal cells compared to

mock-treated and TGF-β-stimulated cells at day 23 on MDS analysis (Figure 5.4), consistent with logFC miRNA expression changes between TGF-β withdrawal cells normalised to TGF-β-stimulated cells and mock-treated cells at all time points of our comparative study (Figures 5.9-5.10).

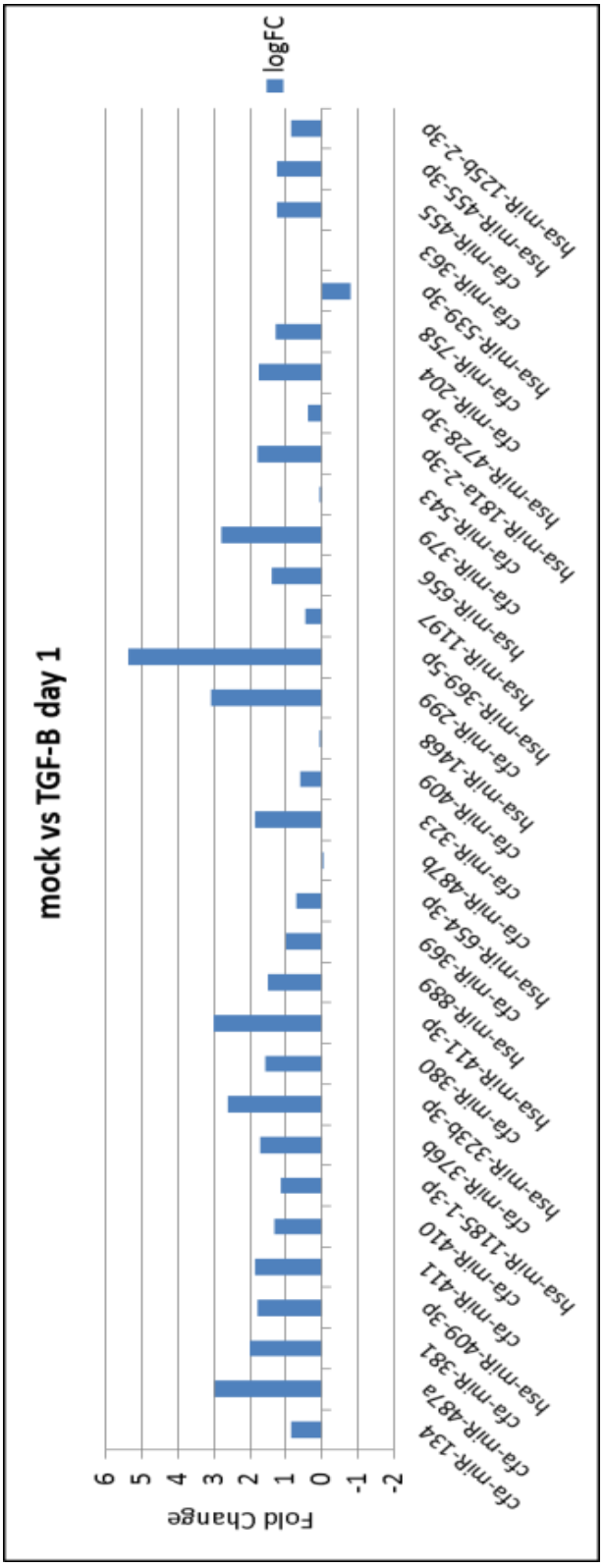

Figure 5.5. Fold change in mapped miRNA expression in REM cells stimulated with TGF-β for 1 day normalised to mock-treated cells at day 1. Log(2) fold change data are presented with normalisation of TGF-β-stimulated cell data to mock-treated cell data. Expression of most mapped miRNAs is upregulated after TGF-β stimulation compared to mock-treated cells.

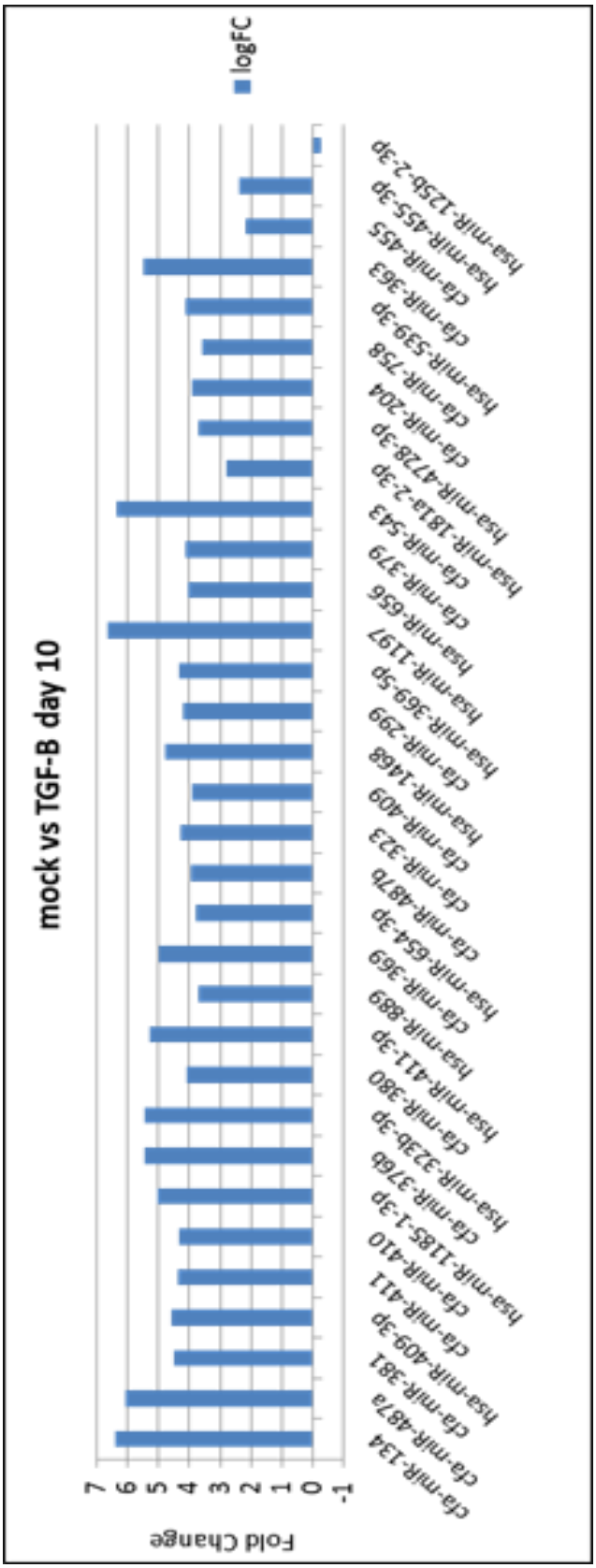

Figure 5.6. Fold change in mapped miRNA expression in REM cells stimulated with TGF-β for 10 days normalised to mock-treated cells at day 10. Log(2) fold change data are presented with normalisation of TGF-β-stimulated cell data to mock-treated cell data. Expression of most mapped miRNAs is upregulated after TGF-β stimulation compared to mock-treated cells.

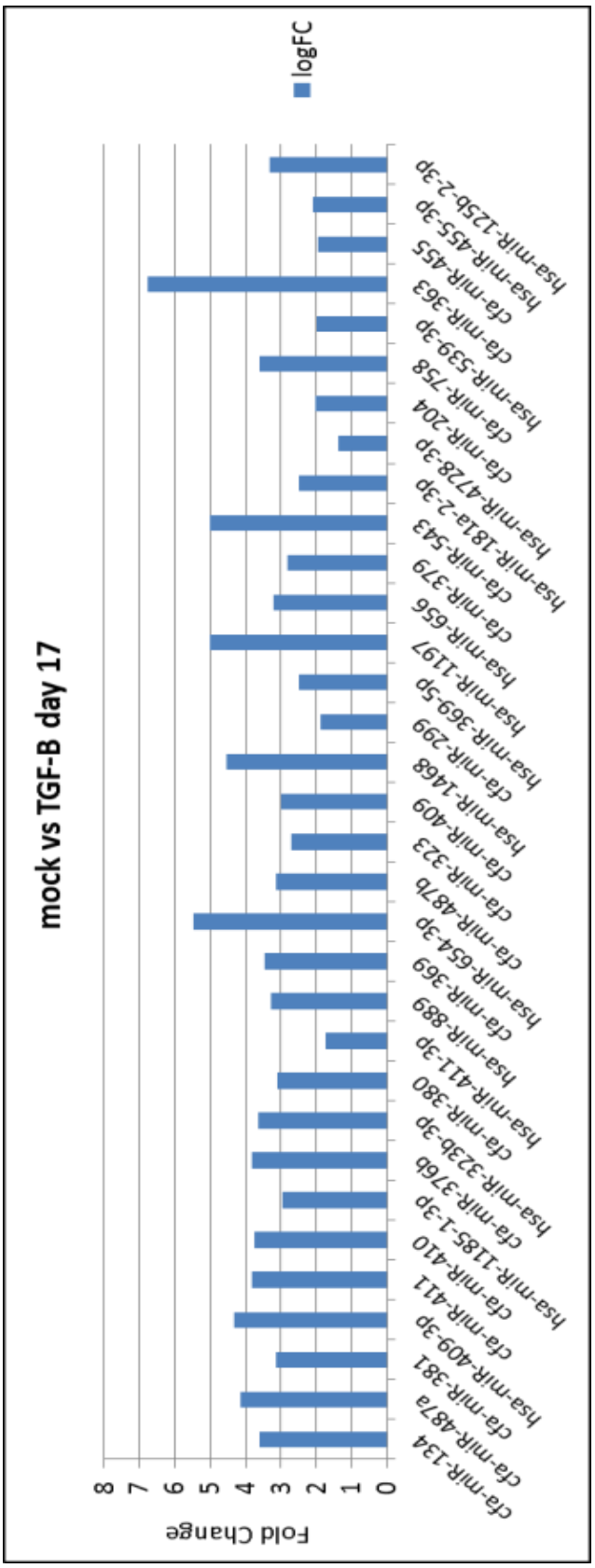

Figure 5.7. Fold change in mapped miRNA expression in REM cells stimulated with TGF-β for 17 days normalised to mock-treated cells at day 17. Log(2) fold change data are presented with normalisation of TGF-β-stimulated cell data to mock-treated cell data. Expression of all mapped miRNAs is upregulated after TGF-β stimulation compared to mock-treated cells.

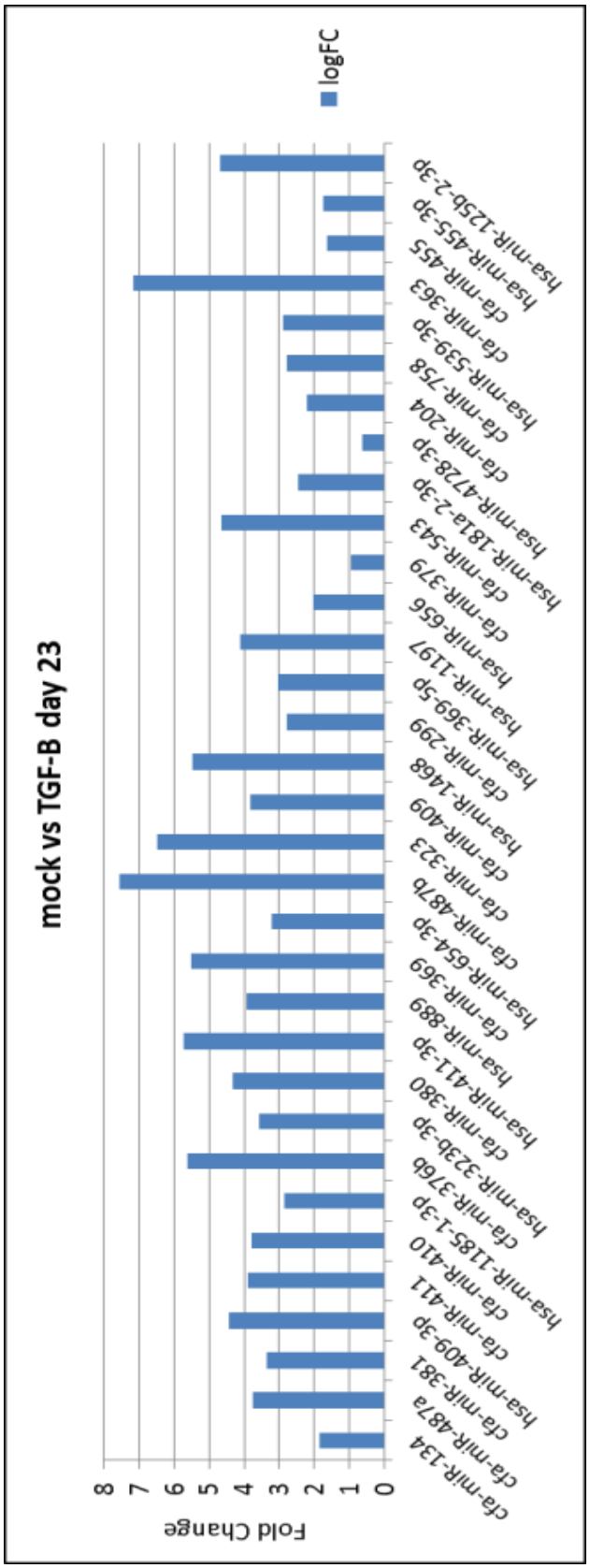

Figure 5.8. Fold change in mapped miRNA expression in REM cells stimulated with TGF-β for 23 days normalised to mock-treated cells at day 23. Log(2) fold change data are presented with normalisation of TGF-β-stimulated cell data to mock-treated cell data. Expression of all mapped miRNAs is upregulated after TGF-β stimulation compared to mock-treated cells

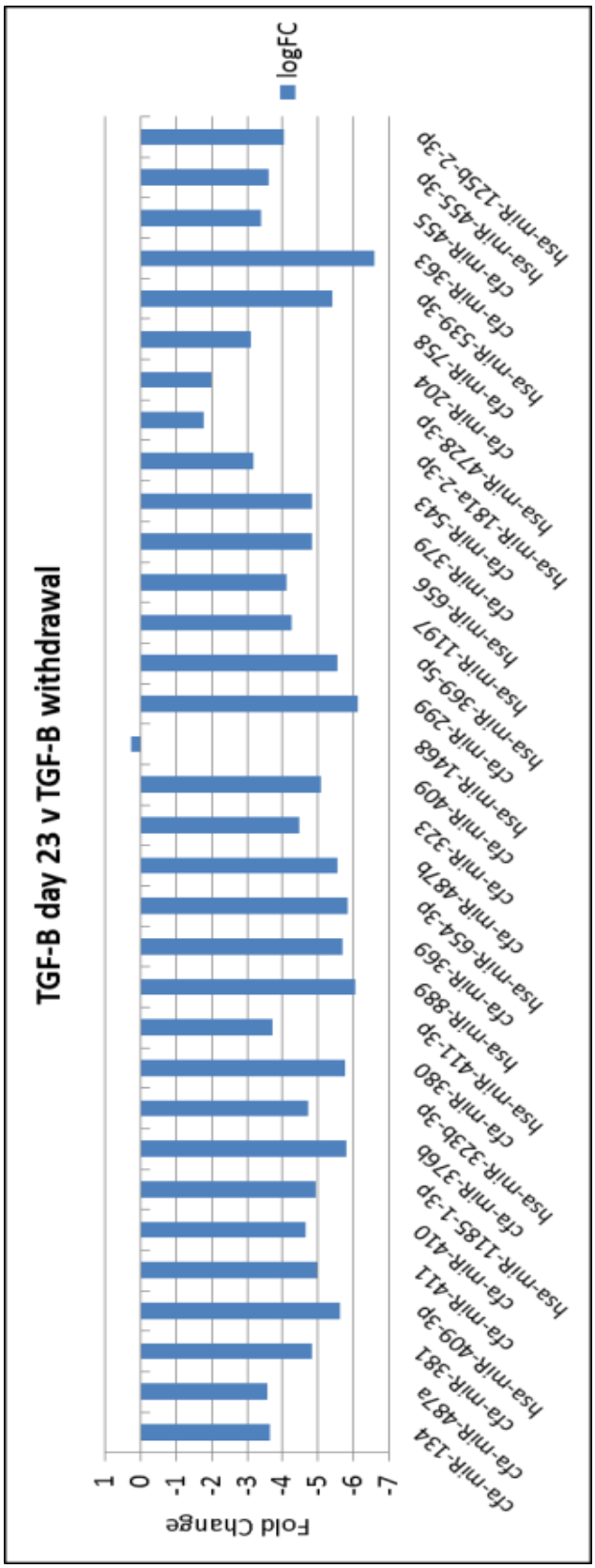

Figure 5.9. Fold change in mapped miRNA expression in REM TGF-β-withdrawal samples normalised to TGF-β-stimulated cells for 23 days. Log(2) fold change data are presented with normalisation of TGF-β-withdrawal cell data to TGF-β-stimulated cell data. Expression of most mapped miRNAs is downregulated TGF-β withdrawal compared to TGF-β-stimulated cells.

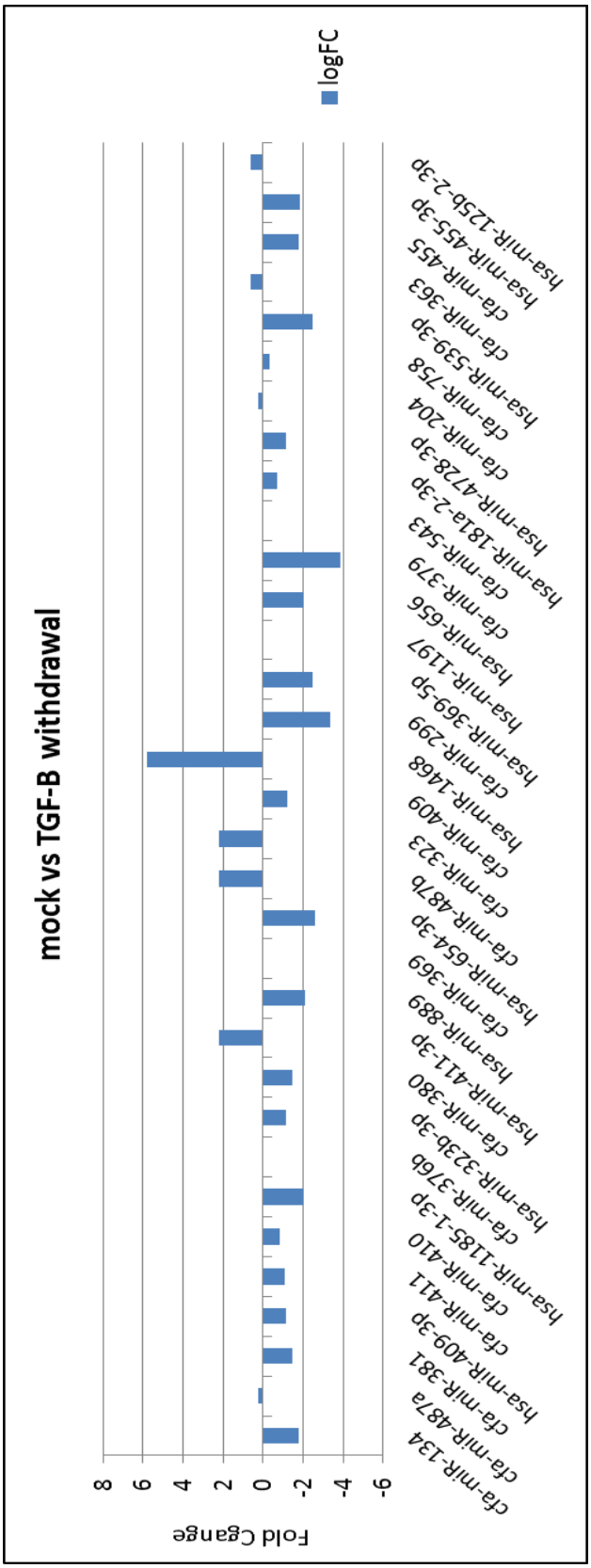

Figure 5.10. Fold change in mapped miRNA expression in REM TGF-β-withdrawal samples normalised to mock-treated cells for 23 days. Four of the 33 miRNAs were not expressed in TGF-β-withdrawal samples. Log(2) fold change data are presented with normalisation of TGF-β-withdrawal cell data to mock-treated cell data. Expression of most mapped miRNAs is downregulated in TGF-β withdrawal samples compared to mock-treated cells.

5.3.5 TGF-β-stimulated cells show significant changes in miRNA expression

For each different time point during this study, we chose only the significant expression changes in specific miRNAs according to the adjusted p-values (FDR) for each mock and TGF-β comparison. Each time point showed different results compared to the other time points. As expected after observing the results from the MDS analysis in Figure 5.4, there were no significant changes according to the FDR (adjusted p-value) of ≤0.05 between mock-treated and TGF-β-stimulated cells after only 1 day of treatment, while those from days 10, 17 and 23 showed significant changes in miRNA expression, some of which were consistent at all time points. Only significant results from calculations for logFC for each time point will be shown. These results are based on TGF-β-stimulated and TGF-β withdrawal cell data normalised to mock-treated cell data, and also TGF-β withdrawal cell data normalised to TGF-β-stimulated cell data.

5.3.5.1 MiRNA expression after 10 days of stimulation with TGF-β

From all the upregulated miRNAs shown above, 29 miRNAs were significantly upregulated in TGF-β-stimulated REM cells compared to mock-treated REM cells harvested at day 10. From these, 16 were known canine miRNAs and 13 were mapped to known human miRNAs as shown in Figure 5.11. There were no downregulated miRNAs found in TGF-β-stimulated cells harvested at day 10 as only positive values were reported.

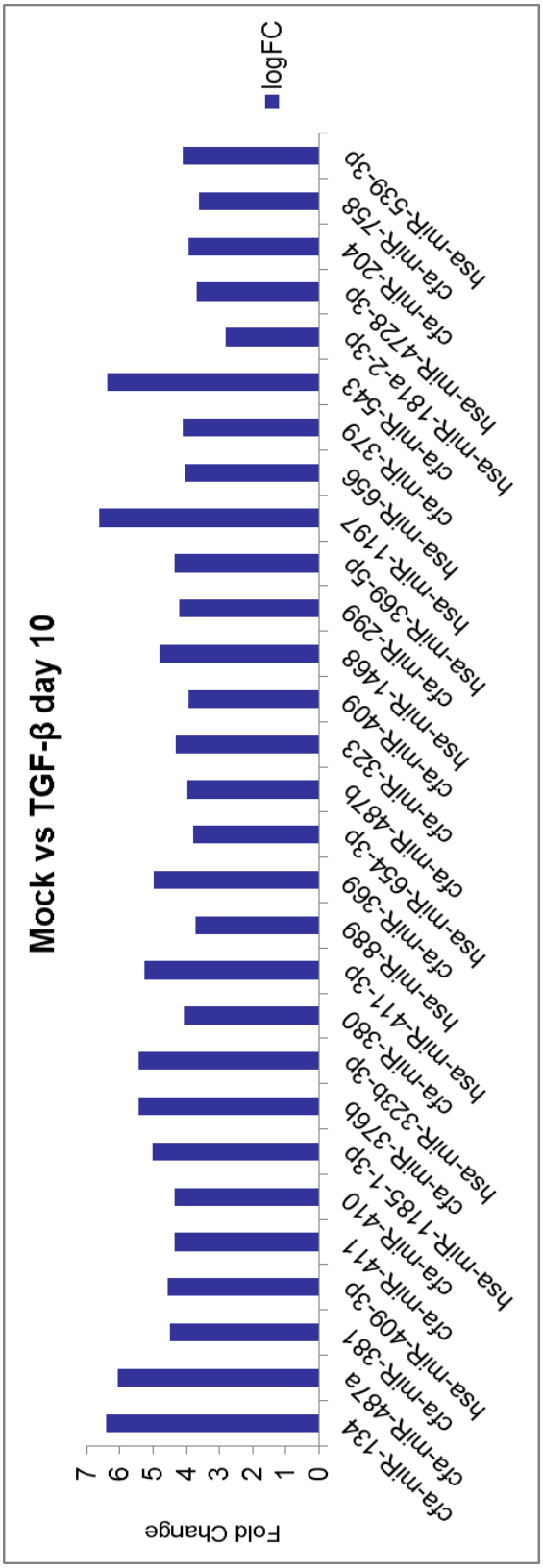

Figure 5.11. Significant fold changes in mapped miRNA expression in REM cells stimulated with TGF-β for 10 days, normalised to mock-treated cells at day 10. Log(2) fold change data are presented with normalisation of TGF-β-stimulated cell data to mock-treated cell data, only significant fold changes are presented (i.e. False Discovery Rate ≤0.05). Expression of all mapped miRNAs is significantly upregulated after TGF-β-stimulation compared to mock-treated cells at day 10.

Furthermore, in order to assess how highly expressed the upregulated miRNAs were compared to each other in the TGF-β-stimulated samples normalised to mock-treated cells after 10 days of stimulation, the logCPM was calculated where, as stated in Chapter 5.3.2, high or low values would show if the miRNAs are highly or lowly expressed, respectively. Here, we show that there are differences in relative levels of expression between the upregulated miRNAs in these samples. Among the 5 miRNAs that showed the highest expression, we observed 2 human (has-miR-181a-2-3p and 409-3p) and 3 canine (cfa-miR-411, 381 and 410) miRNAs as shown in Figure 5.12.

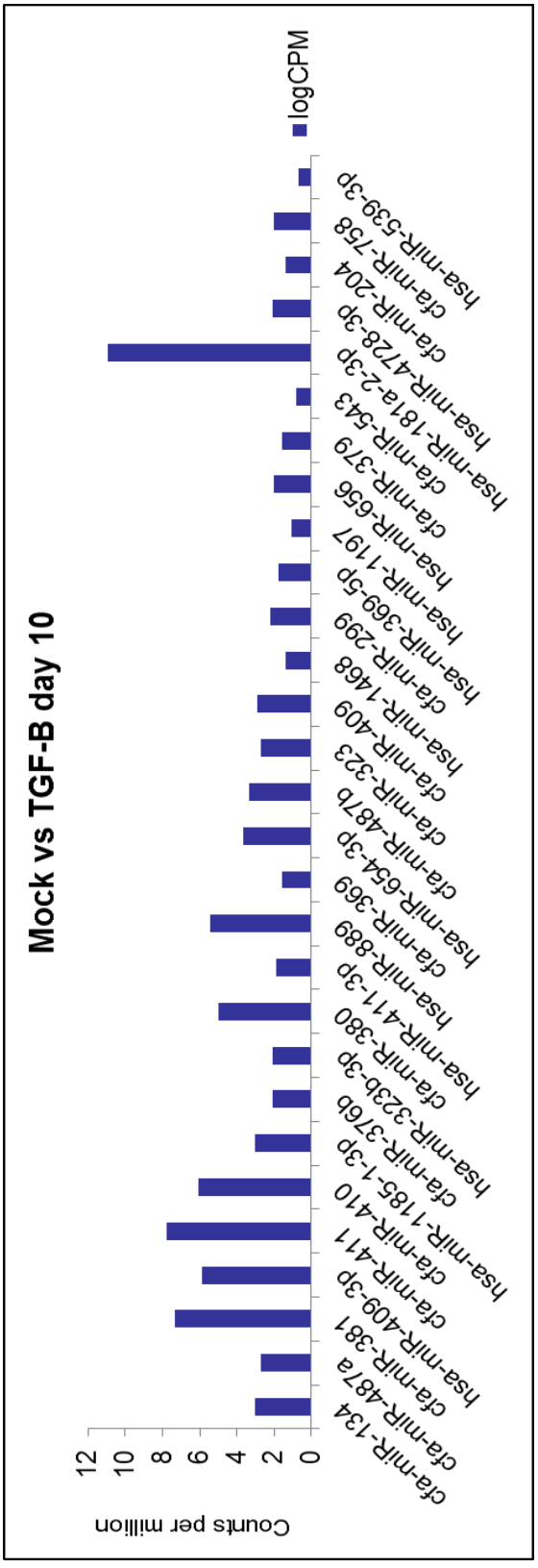

Figure 5.12. Differences in miRNA expression in REM cells stimulated with TGF-β for 10 day, compared to mock-treated cells at day 10. Average log (2) counts per million data are presented (with normalisation of TGF-β-stimulated cell data to mock-treated cell data). According to the fold change data shown in Figure 5.11, only significant data are presented (False Discovery Rate ≤0.05).

5.3.5.2 MiRNA expression after 17 days of stimulation with TGF-β

After 17 days of stimulation with TGF-β, the expression of 11 miRNAs was significantly upregulated when compared to mock-treated cells, including 6 canine and 5 human miRNAs. Similar to the comparative study after 10 days of TGF-β stimulation, no mapped miRNA expression was found to be significantly downregulated in TGF-β-stimulated samples compared to the mock-treated samples as shown in Figure 5.13. Interestingly, 9 out of the 11 significantly upregulated miRNAs at day 17 of TGF-β stimulation were also significantly upregulated after 10 days of treatment (cfa-miR-411, 410, 381, 487a and 380, and hsa-miR-409-3p, 654-3p, 1468 and 889).

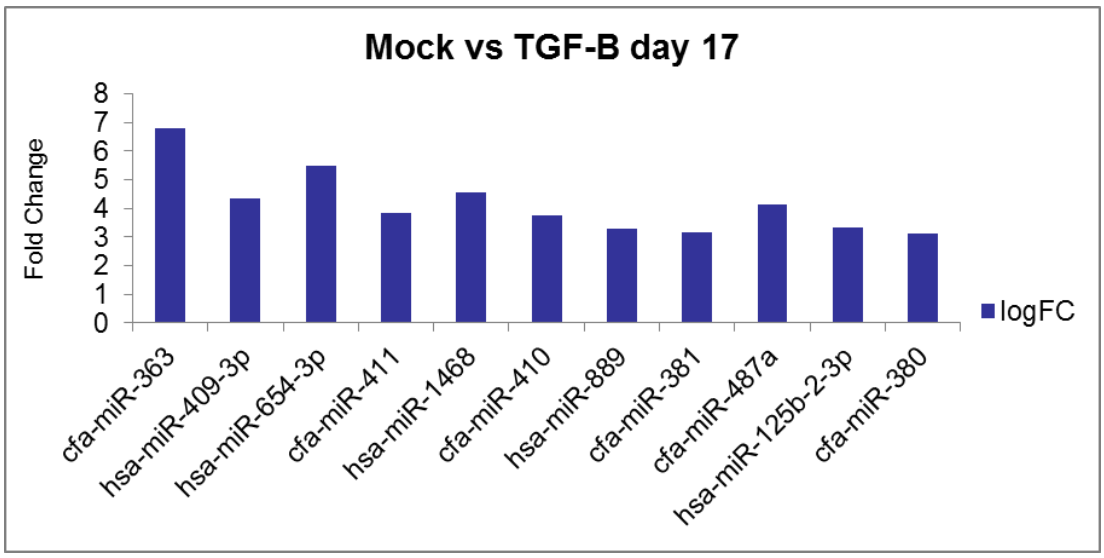

Figure 5.13. Significant fold changes in mapped miRNA expression in REM cells stimulated with TGF-β for 17 days, normalised to mock-treated cells at day 17. Log(2) fold change data are presented with normalisation of TGF-β-stimulated cell data to mock-treated cell data, only significant fold changes are presented (i.e. False Discovery Rate ≤0.05). Expression of all mapped miRNAs is significantly upregulated after TGF-β-stimulation compared to mock-treated cells at day 17.

When expressed in logCPM values the changes in miRNA expression after 17 days of TGF-β stimulation compared to mock-treated cells were similar to those after 10 days of stimulation. Of the 5 most highly expressed miRNAs in cells after 17

days of TGF-β stimulation compared to mock-treated cells, 3 were canine miRNAs (cfa-miR-411, 381 and 410) and 2 were human miRNAs (hsa-miR-409-3p and 889) as shown in Figure 5.14. Comparing these data with those following 10 days of TGF-β stimulation we demonstrated that the 3 canine miRNAs and 1 human miRNA (hsa-miR-409-3p) were amongst the 5 most highly expressed miRNAs in both analyses, even though they are ranked in different order based on their magnitudes of expression on days 10 and 17.

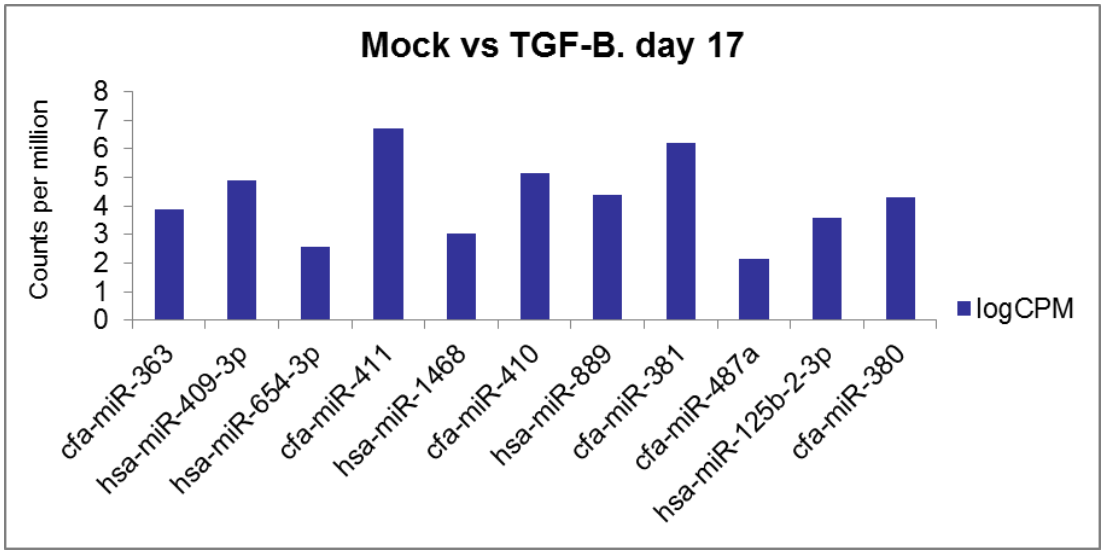

Figure 5.14. Differences in miRNA expression in REM cells stimulated with TGF-β for 17 day, compared to mock-treated cells at day 17. Average log (2) counts per million data are presented (with normalisation of TGF-β-stimulated cell data to mock-treated cell data). According to the fold change data showed in Figure 5.13, only significant data are presented (False Discovery Rate ≤0.05).

5.3.5.3 MiRNA expression after 23 days of stimulation with TGF-β

After 23 days of stimulation with TGF-β, the expression of 12 miRNAs was significantly upregulated compared with mock-treated REM cells. Eight of these upregulated miRNAs were canine and only 4 were human as shown in Figure 5.15. Of these 12 upregulated miRNAs, 9 were also upregulated in cells after 17 days of TGF-β stimulation. The miRNAs significantly upregulated at both days 17 and 23

included 5 canine (cfa-miR-363, 411, 410, 381 and 380) and 4 human (hsa-miR-409-3p, 1468, 889 and 125b-2-3p) miRNAs. Likewise, 10 of the 12 significantly upregulated miRNAs after 23 days of TGF-β stimulation were also significantly upregulated after 10 days of TGF-β stimulation. Overall, there were 7 miRNAs significantly upregulated following TGF-β stimulation at the 3 different time points, including 4 canine (cfa-miR-411, 410, 381 and 380) and 3 human (hsa-miR-409.3p, 1468 and 889) miRNAs.

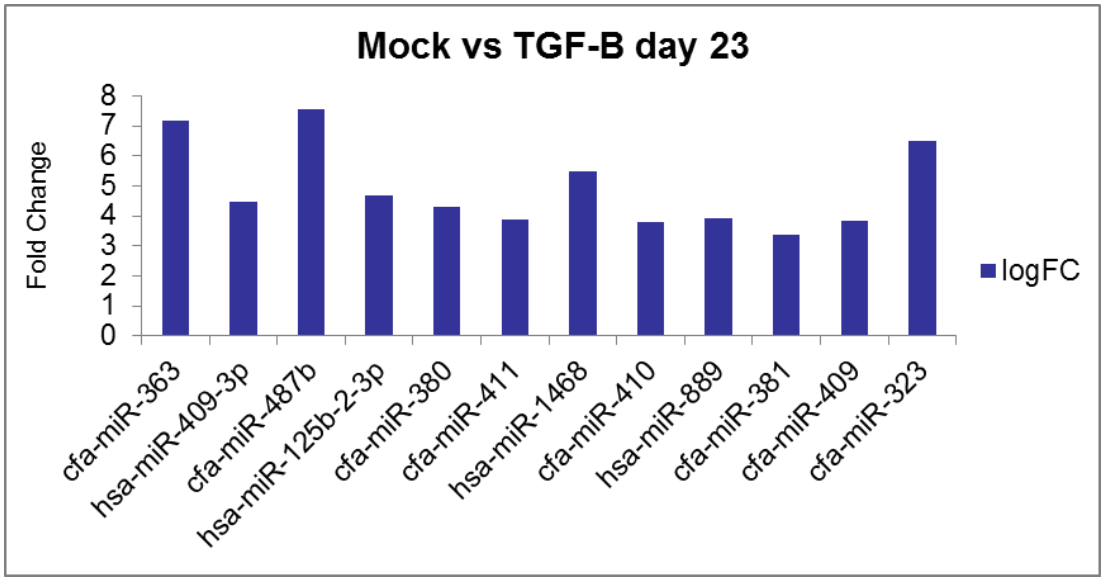

Figure 5.15. Significant fold changes in mapped miRNA expression in REM cells stimulated with TGF-β for 23 days, normalised to mock-treated cells at day 23. Log(2) fold change data are presented with normalisation of TGF-β-stimulated cell data to mock-treated cell data, only significant fold changes are presented (i.e. False Discovery Rate ≤0.05). Expression of all mapped miRNAs is significantly upregulated after TGF-β-stimulation compared to mock-treated cells at day 23.

As stated above, there were 7 miRNAs consistently significantly upregulated in TGF-β-stimulated cells after the three different time points. When expressed as logCPM values for day 23 after TGF-β stimulation, we observed that 4 of the 5 most highly expressed miRNAs compared with mock-treated cells, were also amongst the 5 most highly expressed miRNAs after 10 and 17 days of TGF-β stimulation (Figure 5.16). The 5 most highly expressed miRNAs at day 23 were human has-miR-409-3p

and canine cfa-miR-411, 381, 410 and 363, the latter being the only miRNA not to be also highly expressed after days 10 and 17 of TGF-β stimulation.

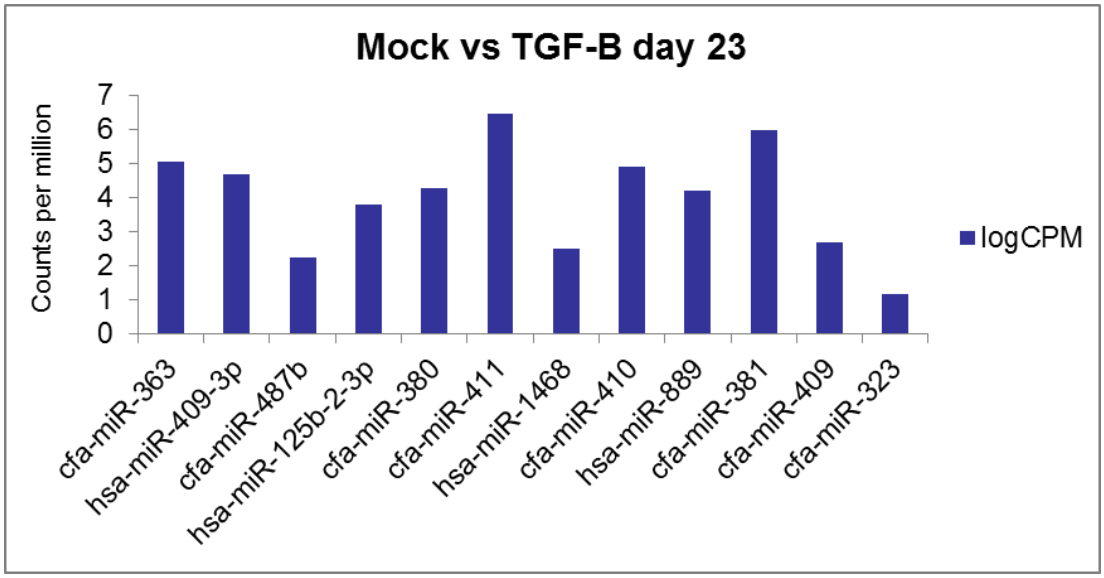

Figure 5.16. Differences in miRNA expression in REM cells stimulated with TGF-β for 23 day, compared to mock-treated cells at day 23. Average log (2) counts per million data are presented (with normalisation of TGF-β-stimulated cell data to mock-treated cell data). According to the fold change data showed in Figure 5.15, only significant data are presented (False Discovery Rate ≤0.05).

5.3.5.4 MiRNA expression comparison between mock-treated cells and cells stimulated with TGF-β for 19 days prior to withdrawal until day 23

There were not many significant differences in miRNA expression between mock-treated cells and cells stimulated with TGF-β for 19 days and harvested on day 23 (4 days after TGF-β withdrawal), the only upregulated miRNA in the TGF-β withdrawal sample was hsa-miR-1468 (Figure 5.17). Interestingly, this human miRNA was also upregulated in all time points described above. TGF-β withdrawal cells would be expected to return to a regular epithelial phenotype, exhibited by the mock-treated cells after TGF-β withdrawal, although there are no studies

demonstrating how long canine mammary carcinoma cells would take to go back to an epithelial phenotype after TGF-β withdrawal.

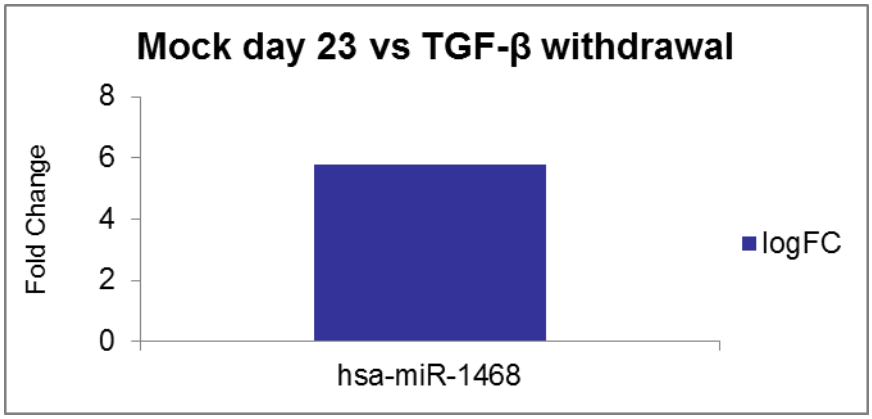

Figure 5.17. Significant fold change in mapped miRNA expression in REM cells from which TGF-β was withdrawn after 19 days and harvested for analysis 4 days later, normalised to mock-treated cells at day 23. Log(2) fold change data are presented with normalisation of TGF-β withdrawal cell data to mock-treated cell data, only significant fold changes are presented (i.e. False Discovery Rate >0.05). Expression of only one mapped miRNA is significantly upregulated after TGF-β withdrawal compared to mock-treated cells at day 23.

As this human miRNA was the only upregulated miRNA in this study, we do not have a way to assess how highly expressed it was compared to other miRNAs within the same comparative study, however, if we compare it to upregulated miRNAs from different time points, we can observe that its logCPM value, as shown in Figure 5.18, is not as highly expressed as any of the 5 most highly expressed miRNAs at the other time points described above.

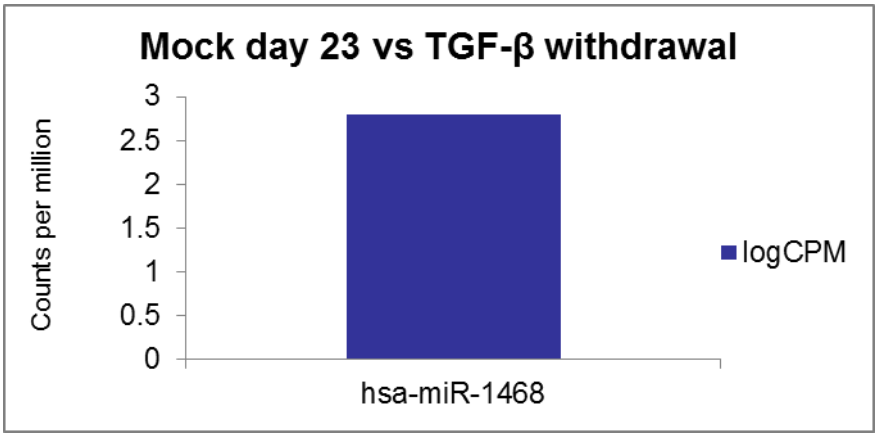

Figure 5.18. Difference in mapped miRNA expression in REM cells from which TGF-β was withdrawn after 19 days and harvested for analysis 4 days later, normalised to mock-treated cells at day 23. Average log (2) counts per million data are presented (with normalisation of TGF-β-stimulated cell data to mock-treated cell data). According to the fold change data showed in Figure 5.17, only significant data are presented (False Discovery Rate ≤0.05). Expression of only one miRNA is significantly increased after TGF-β withdrawal compared to mock-treated cells.

5.3.5.5 MiRNA expression comparison between TGF-β-stimulated and TGF-β withdrawal samples

When the miRNA expression profile of cells stimulated with TGF-β for 23 days was compared with that of cells from which TGF-β was withdrawn from day 19 to 23, several of the miRNAs observed to be significantly upregulated during TGF-β stimulation (Figures 5.13, 5.15, 5.17) were downregulated on TGF-β withdrawal cells (Figure 5.19).

In this case, TGF-β-stimulated cell data were used to normalise the TGF-β withdrawal cell data. The significantly upregulated miRNAs found when TGF-β-stimulated cell data were normalised with mock-treated cell data at 10, 17, and 23 days of TGF-β stimulation, and the near total lack of significant miRNA expression changes when withdrawal cells were compared to mock-treated cells.

All the significant changes in miRNA expression were downregulations, and none were found to be significantly upregulated as shown in Figure 5.19. Significant

fold changes in mapped miRNA expression in REM cells from which TGF-β was withdrawn after 19 days and harvested for analysis 4 days later, normalised to cells stimulated with TGF-β for 23 days. Log(2) fold change data are presented with normalisation of TGF-β withdrawal cell data to TGF-β-stimulated cell data, only significant fold changes are presented (i.e. False Discovery Rate >0.05). Expression of all mapped miRNAs is significantly downregulated after TGF-β withdrawal compared to TGF-β-stimulated cells at day 23.. Half of the 16 significantly downregulated miRNAs were canine and half were human miRNAs. Six of these significantly downregulated miRNAs in TGF-β withdrawal cells were upregulated in TGF-β-stimulated samples in all the time points previously shown. These miRNAs were the canine cfa-miR-411, 410, 381 and 380 and the human hsa-miR-409-3p and 889.

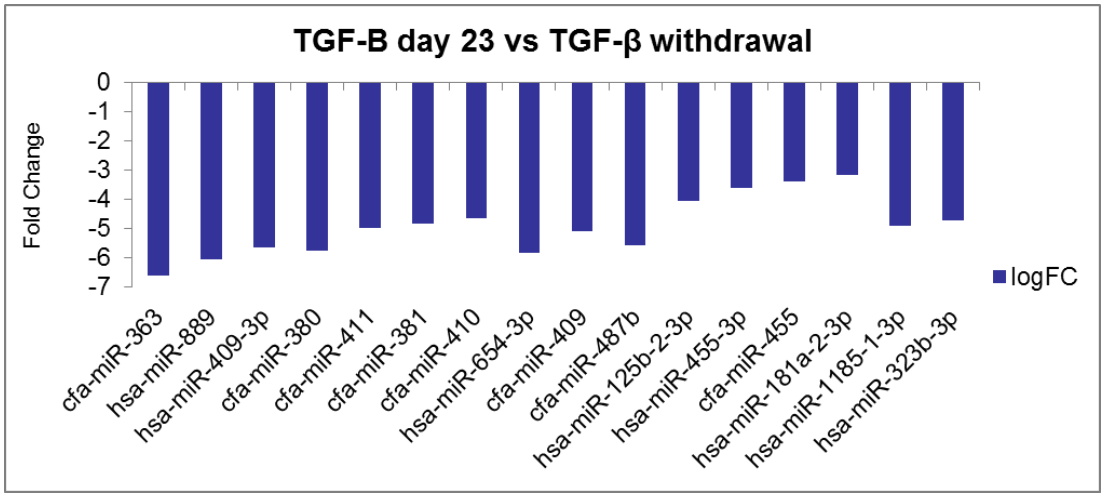

Figure 5.19. Significant fold changes in mapped miRNA expression in REM cells from which TGF-β was withdrawn after 19 days and harvested for analysis 4 days later, normalised to cells stimulated with TGF-β for 23 days. Log(2) fold change data are presented with normalisation of TGF-β withdrawal cell data to TGF-β-stimulated cell data, only significant fold changes are presented (i.e. False Discovery Rate >0.05). Expression of all mapped miRNAs is significantly downregulated after TGF-β withdrawal compared to TGF-β-stimulated cells at day 23.

When expressed using logCPM values among the 5 most profoundly differently expressed miRNAs in withdrawal cells, canine cfa-miR-411 and 381 were also highly expressed following TGF-β stimulation for 10, 17 and 23 days (Figures 5.12, 5.13 and 5.16). Human miRNA hsa-miR-181a-2-3p showed the highest

expression in withdrawal cells and was also highly expressed in cells stimulated with TGF-β for 10 days.

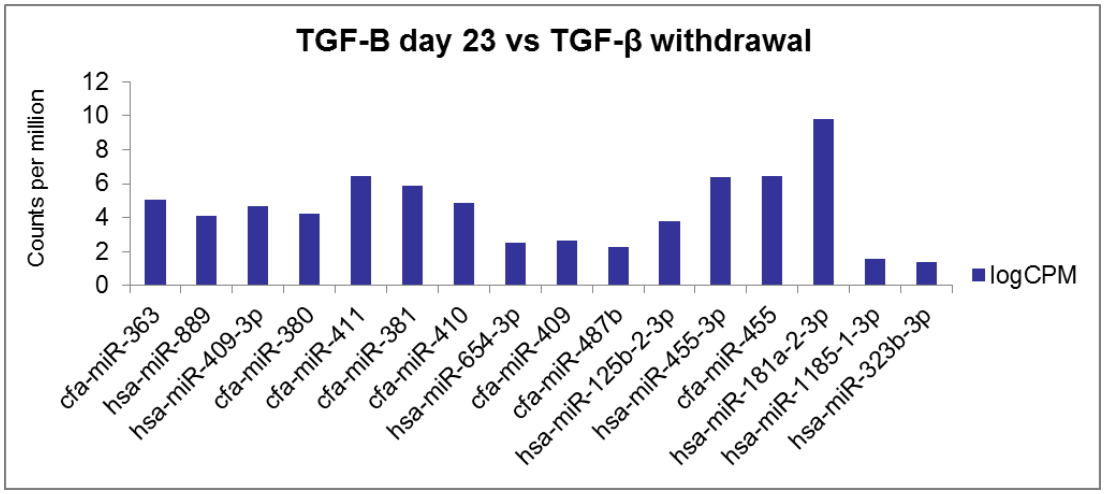

Figure 5.20. Differences in mapped miRNA expression in REM cells from which TGF-β was withdrawn after 19 days and harvested for analysis 4 days later, normalised to cells stimulated with TGF-β for 23 day. Average log (2) counts per million data are presented (with normalisation of TGF-β-stimulated cell data to mock-treated cell data). According to the fold change data showed in Figure 5.19, only significant data are presented (False Discovery Rate ≤0.05). Expression of all mapped miRNAs is significantly increased after TGF-β withdrawal compared to TGF-β-stimulated cells.

5.3.6 Canine miRNAs with the most significant changes

From all the significantly upregulated or downregulated miRNAs throughout the various time points analysed, 6 of them (canine cfa-miR-411, 410, 381 and 380; and human hsa-miR-409-3p and 889) showed similar changes at all time points. These data are presented in Figure 5.21where all miRNAs are significantly upregulated after TGF-β stimulation at days 10, 17 and 23; and significantly downregulated after TGF-β withdrawal when compared to contemporaneous mock-treated cells. These data are also presented as a table (Table 5.2) showing the fold change difference of each miRNA expression at these different time-points after TGF-β stimulation and after TGF-β withdrawal. Only results with adjusted p-values

or false discovery rate (FDR) of ≤ 0.05 showing significant changes among the different samples were taken into account.

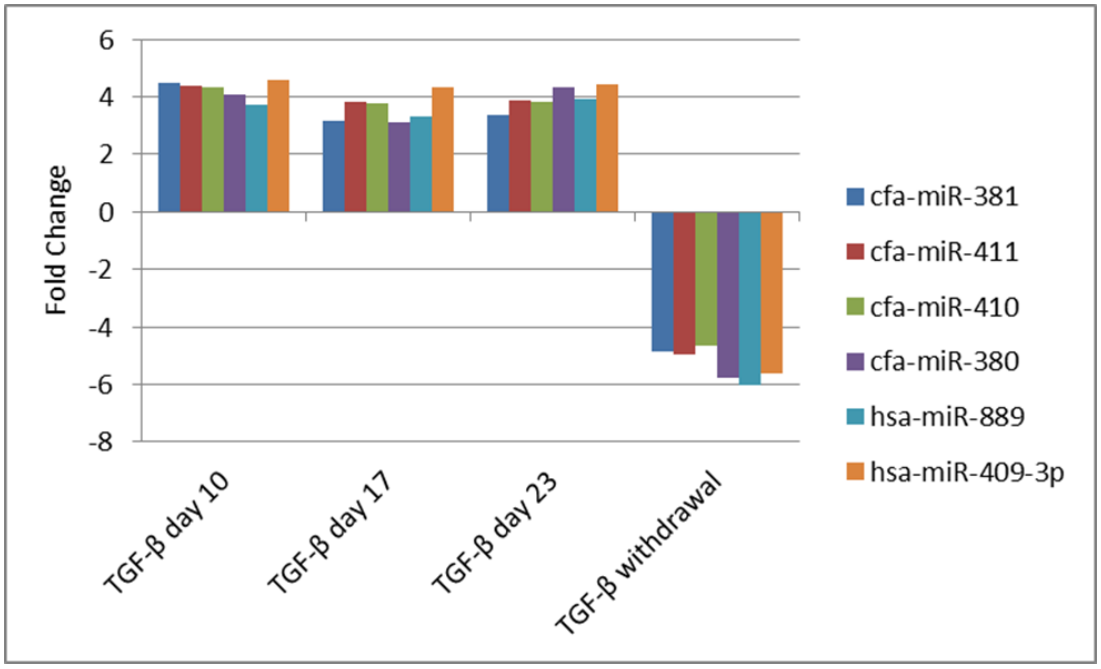

Figure 5.21. Consistent significant changes in miRNA expression in REM cells exposed to TGF-β at various time points. There is a remarkable upregulation in fold change of cfa-miR-381, 411, 410, 380, has-miR-889 and has-miR-409-3p at days 10, 17 and 23; whereas in the TGF-β withdrawal samples they were all downregulated. All data are normalised to mock-treated cells.

miRNA	TGF-β day 10 **	TGF-β day 17 ***	TGF-β day 23 ***	TGF-β withdrawal **
cfa-miR-381	4.486478673	3.145995686	3.372973206	-4.848036247
cfa-miR-411	4.365261163	3.832102958	3.879174972	-4.983495066
cfa-miR-410	4.348743275	3.761908393	3.798544273	-4.649481567
cfa-miR 380	4.064768987	3.116346566	4.313203417	-5.771822492
hsa-miR-889	3.710135129	3.297134688	3.924071407	-6.048847438
hsa-miR-409-4p	4.57710827	4.340934776	4.447358314	-5.635364374

Table 5.2. Fold change differences in the expression of several miRNAs at different time points of TGF-β stimulation of REM cells. At days 10, 17 and 23 of TGF-β stimulation, cells show a significant increase in the expression of cfa-miR-381, 411, 410, 380, hsa-miR-889 and hsa-miR-409-3p. REM cells from which TGF-β was withdrawn after 19 days and harvested for analysis 4 days later expressed significant decreases in these mRNAs when compared to cells continuously

stimulated with TGF-b for 23 days. ** = False discovery rate ≤ 0.005, * = False discovery rate ≤ 0.05**

5.3.7 MicroRNA validation assays

Considering that validation is needed for the confirmation of these global miRNA analysis findings, we carried out specific miRNA expression assays targeting the mature sequences of the 4 canine miRNAs that the expression of which were consistently similarly altered on TGF-β stimulation throughout this study (cfa-miR-411, 410, 381 and 380). As described in Chapter 2.13, we performed qRT-PCR targeting the specific mature sequences of these miRNAs, which all showed a peak in expression by day 10 of treatment with TGF-β and they were also downregulated in the TGF-β withdrawal sample. All fold changes of miRNA expression were normalised to reference gene RPL32 as specified in Chapter 2.7.7. The results of the specific miRNA analyses were therefore consistent with the global miRNA expression analysis patterns, confirming our findings are relevant and further investigation in this field is warranted. The specific qRT-PCR expression data for cfa-miR-380, cfa-miR-381, cfa-miR-410 and cfa-miR-411 are presented in Figure 5.22, Figure 5.23, Figure 5.24 and Figure 5.25, respectively.

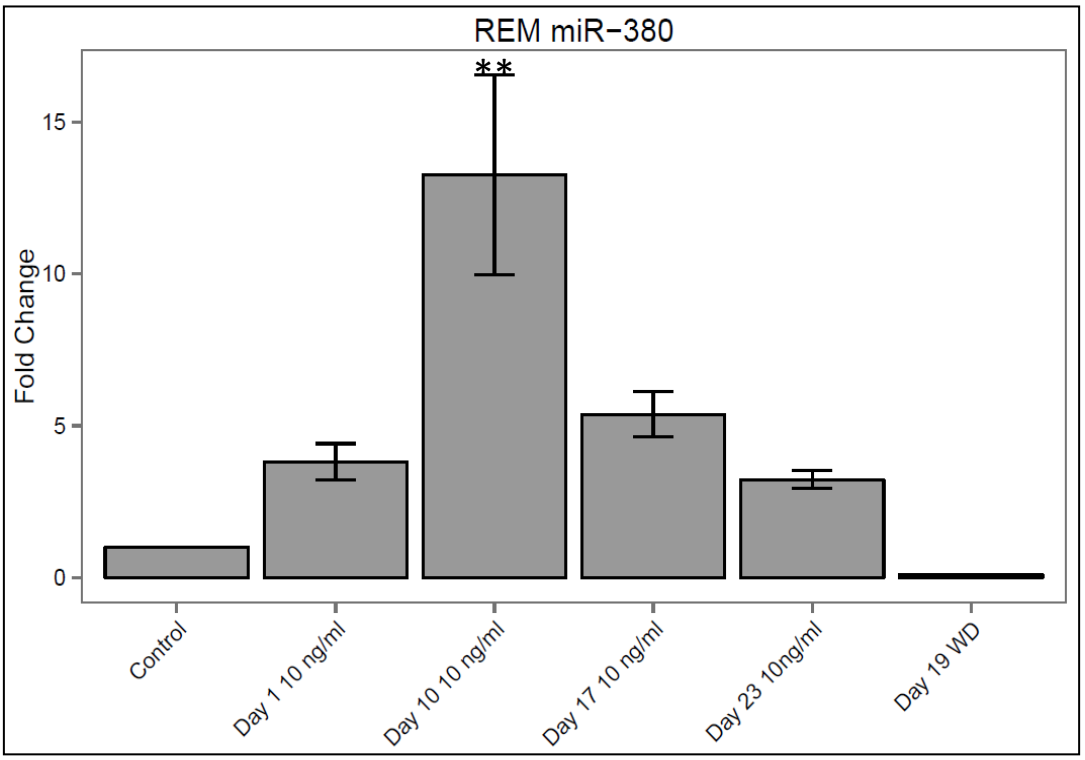

Figure 5.22. Canine miRNA-380 expression is increased after TGF-β stimulation of REM cells, and decreases after its withdrawal, assessed by specific qRT-PCR. As observed in global miRNA analyses, peak expression was observed at day 10 of TGF-β stimulation and decreased thereafter. Control = untreated cells harvested at day 23, Day 1 10 ng/ml = cells harvested at day 1 of TGF-β treatment, Day 10 10 ng/ml = cells harvested at day 10 of TGF-β treatment, Day 17 10 ng/ml = cells harvested at day 17 of TGF-β treatment, Day 23 10 ng/ml = cells harvested at day 23 of TGF-β treatment, Day 19 WD = cells harvested at day 23 after TGF-β withdrawal at day 19. P values comparing stimulated cells with control cells harvested at day 23: ** = p value ≤ 0.005

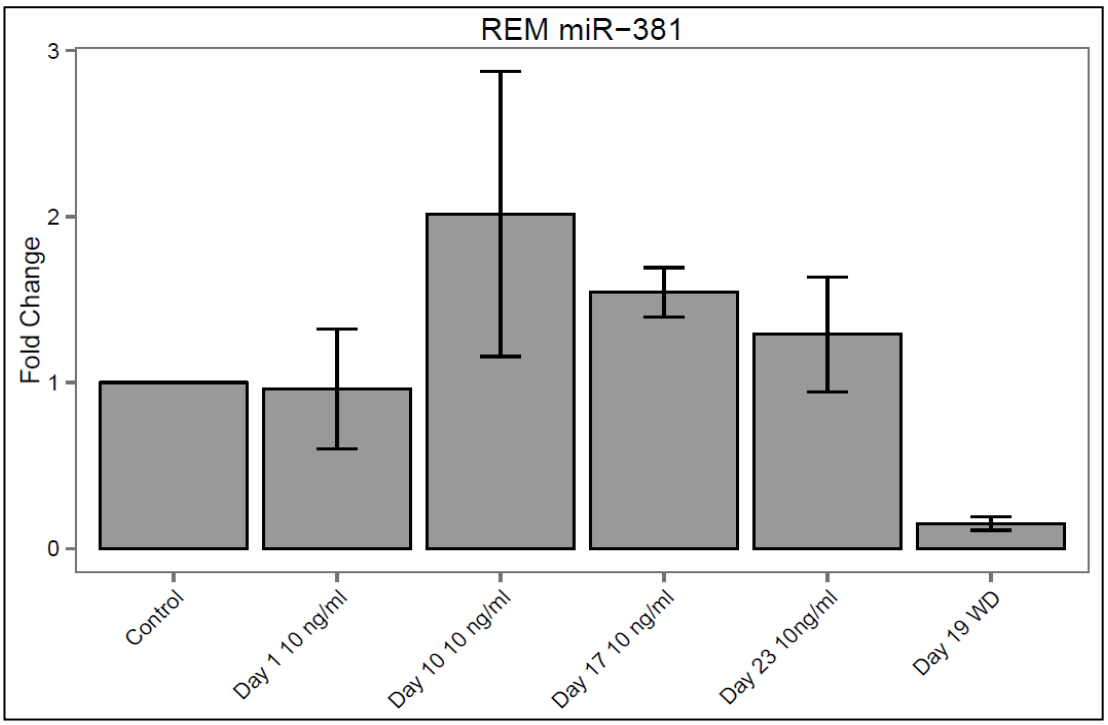

Figure 5.23. Canine miRNA-381 expression is increased after prolonged TGF-β stimulation of REM cells, and decreases after its withdrawal, assessed by specific qRT-PCR. As observed in global miRNA analyses, peak expression was observed at day 10 of TGF-β stimulation and decreased thereafter. Control = untreated cells harvested at day 23, Day 1 10 ng/ml = cells harvested at day 1 of TGF-β treatment, Day 10 10 ng/ml = cells harvested at day 10 of TGF-β treatment, Day 17 10 ng/ml = cells harvested at day 17 of TGF-β treatment, Day 23 10 ng/ml = cells harvested at day 23 of TGF-β treatment, Day 19 WD = cells harvested at day 23 after TGF-β withdrawal at day 19. There were no significant changes.

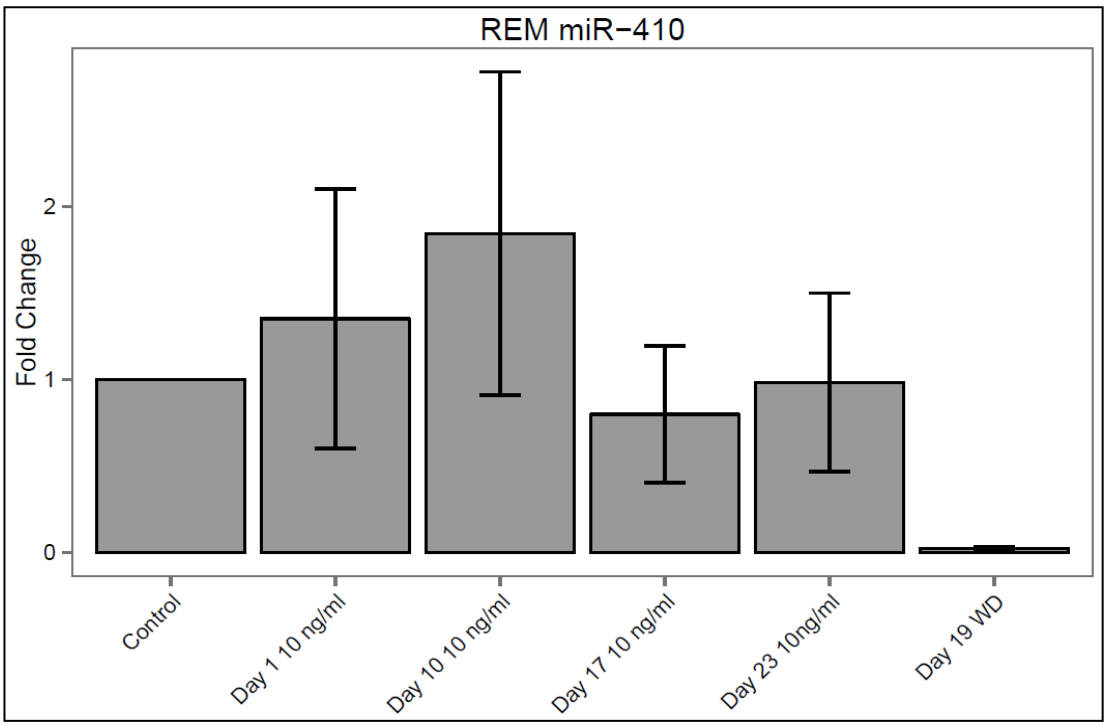

Figure 5.24. Canine miRNA-410 expression is increased after short term TGF-β stimulation of REM cells, and decreases after its withdrawal, assessed by specific qRT-PCR. As observed in global miRNA analyses, peak expression was observed at day 10 of TGF-β stimulation and decreased thereafter. Control = untreated cells harvested at day 23, Day 1 10 ng/ml = cells harvested at day 1 of TGF-β treatment, Day 10 10 ng/ml = cells harvested at day 10 of TGF-β treatment, Day 17 10 ng/ml = cells harvested at day 17 of TGF-β treatment, Day 23 10 ng/ml = cells harvested at day 23 of TGF-β treatment, Day 19 WD = cells harvested at day 23 after TGF-β withdrawal at day 19. There were no significant changes.

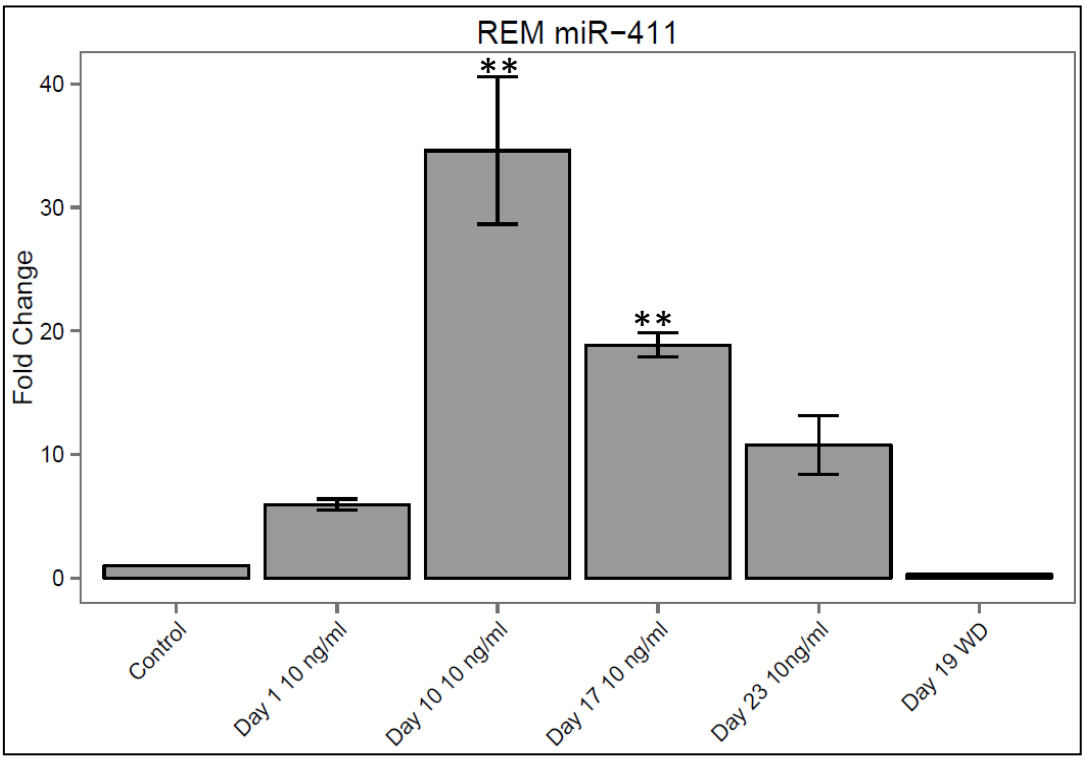

Figure 5.25. Canine miRNA-411 expression is increased after TGF-β stimulation of REM cells, and decreases after its withdrawal, assessed by specific qRT-PCR. As observed in global miRNA analyses, peak expression was observed at day 10 of TGF-β stimulation and decreased thereafter. Control = untreated cells harvested at day 23, Day 1 10 ng/ml = cells harvested at day 1 of TGF-β treatment, Day 10 10 ng/ml = cells harvested at day 10 of TGF-β treatment, Day 17 10 ng/ml = cells harvested at day 17 of TGF-β treatment, Day 23 10 ng/ml = cells harvested at day 23 of TGF-β treatment, Day 19 WD = cells harvested at day 23 after TGF-β withdrawal at day 19. P values comparing stimulated samples with control cells harvested at day 23: ** = p value ≤ 0.005.

5.4 Discussion

MiRNAs are key regulators of fundamental physiological cell processes, such as tissue differentiation, and in the progression of several diseases like cancer (Foubert, et al., 2010). In cancer they have been shown to have important roles in promotion and suppression of oncogenes (Croce, 2009) as described in Chapter 1.15. After analysing all our samples in an MDS plot (Figure 5.4), we still needed to answer many questions in regards to what properties some of the depicted points were sharing, and in what ways these points differ from each other. This type of analysis is quite useful to discover the tendency of variation between our data, nevertheless, it should not be considered significant prior to a complete comparative study (Coxon et al., 1982b). In order to elucidate which properties our samples shared between each other, we assessed the miRNA expression profiles in a comparative study between mock-treated, TGF-β-stimulated and TGF-β withdrawal cells at different time points. We have shown four key miRNAs are associated with TGF-β-induced EMT of canine mammary carcinoma cells. Both in global miRNA analyses and in specific miRNA-targeted qRT-PCR of TGF-β-stimulated cells at days 10, 17 and 23, mock-treated cells and TGF-β withdrawal cells, we identified 4 canine miRNAs (cfa-miR-411, 381, 410 and 380) which were consistently similarly changed in their expression.

According to the adjusted p-values or false discovery rate (FDR), these miRNAs showed significant changes among the different samples at days 10, 17, and 23 of TGF-β stimulation. In contrast to the TGF-β-stimulated cells, after withdrawal of TGF-β, cells displayed a decrease in the expression of these miRNAs, suggesting that the miRNA expression changes that are associated with a mesenchymal phenotype after TGF-β stimulation are reversed on TGF-β withdrawal. Further investigation is needed to assess the time required for cells to return to their epithelial phenotype after TGF-β withdrawal. *Tian et al., 2013* showed that after exogenous TGF-β withdrawal, Madin-Darby canine kidney epithelial cells might be able to maintain a stable mesenchymal phenotype if autocrine TGF-β signalling is sustained, which can be achieved by stimulation with large doses of exogenous TGF-β (3 ng/ml) (Gal et al., 2013). However, they mention that the irreversibility of EMT is

cell-type-dependent. This is in line with what *Gal et al., 2008* previously demonstrated after TGF-β withdrawal in normal murine mammary epithelia after being exposed to 5 ng/ml of TGF-β for up to 3 weeks, and after confirming that cells acquired a mesenchymal phenotype assessed by morphological and molecular changes. These cells gradually reverted to epithelial monolayers in 2D cultures within 2-12 days after TGF-β withdrawal, and also re-expressed epithelial markers and strongly reduced mesenchymal marker expression levels (Gal et al., 2008). It is not well understood how different cell types respond to TGF-β stimulation and withdrawal regarding EMT- and MET-associated changes and this warrants further investigation with specific cell types, including canine and feline mammary carcinoma cell lines.

Studies in human cancer have shown contrasting results to the data presented here (Swarbrick et al., 2010, Beltran et al., 2011, Snowdon et al., 2011, van Schooneveld et al., 2012, Liu et al., 2013, Torres et al., 2013). *Van Schooneveld et al, 2012* determined that human miR-411 is downregulated in breast cancer samples, using tissue and blood samples obtained from breast cancer patients and healthy volunteers to compare miRNA expression. The breast cancer patients were divided into 3 different groups with localised breast cancer, metastatic breast cancer under treatment, and metastatic breast cancer without treatment. They compared miRNA expression in tumour samples between the breast cancer patients groups and the healthy breast samples, and found that miR-411 was one of the downregulated miRNAs with greater expression in the tumour samples. Interestingly, they observed a similar pattern when analysing serum samples, whereby miR-411 was among other miRNAs which were significantly downregulated in breast cancer patients compared to healthy volunteers. Moreover, they compared the expression of the most significantly downregulated miRNAs in serum samples of patients with metastatic breast cancer under treatment, patients with metastatic breast cancer without treatment, and healthy volunteers. They observed that the expression of miR-411 along with 3 other miRNAs was lower in untreated metastatic breast cancer, compared to samples from patients under treatment, which exhibited a similar miRNA expression to the healthy volunteers samples (van Schooneveld et al., 2012).

Similarly, *Liu et al, 2013* compared miRNA expression in treatment-naive tissue and blood samples from breast cancer patients and healthy donors. They identified one miRNA that was significantly upregulated in expression (miR-155) and 9 other miRNAs significantly downregulated, including miR-381 (Liu et al., 2013). In this case, they did not compare breast cancer samples at different metastatic stages.

Although in the studies by van *Schooneveld et al, 2012* and *Liu et al, 2013* described above human miR-411 and miR-381 expression was downregulated in breast cancer and metastatic breast cancer samples compared to healthy tissues; in our study, canine miR-411 and miR-381 were upregulated in the TGF-β-stimulated canine mammary epithelial cancer cells compared to mock-treated cells *in vitro*. In previous chapters herein, we showed that REM cells morphologically demonstrate an EMT process after TGF-β stimulation and acquire greater migratory capabilities, and at similar time points in this chapter we found upregulation of canine miR-411 and -381, among others, perhaps related to miRNA expression changes after TGF-β-induced EMT.

Moreover, human miR-410 was found to be decreased in expression in human endometrioid endometrial carcinoma tissues when compared to control samples from patients with a precursor lesion (complex atypical hyperplasia) in a previous study (Snowdon et al., 2011), and similarly, a different group compared tissue and blood samples of patients with endometrial cancer and healthy volunteers finding that miR-410 and a pool of other miRNAs were significantly downregulated in cancer patients (Torres et al., 2013).

These conflicting results for cfa-miR-411 and cfa-miR-381 expression in our study and the human miRNAs in the reports presented above might be explained by our study being an *in vitro* study focussed on miRNA expression after TGF-β-induced EMT in canine mammary carcinoma in which metastasis was not assessed. The human studies assessed miRNA expression in human cancers but without assessing EMT-associated changes after TGF-β stimulation. In order to acquire a better understanding of how these specific miRNAs act in canine mammary carcinoma metastasis, we could assess their expression in mammary tissues from

healthy donors and compare them with primary breast cancer tissues and metastatic tissues, with and without treatment. This could allow us assess how well correlated our findings are with those from human cancer research (van Schooneveld et al, 2012 and Liu et al, 2013). As previously mentioned, TGF-β has been shown to have paradoxical activities in cancer progression where it can act as a context-specific tumour suppressor gene or as a tumour promoter (Levy and Hill, 2005). MicroRNAs have also been found to act differently in context-specific environments, as reviewed by *Boggs et al., 2008*. They show that several miRNAs, including miR-17-5p, miR-181b, miR-155 and let-7f, may act as tumour suppressor genes and/or oncogenes depending on their microenvironmental context within different types of tumours, such as mammary tumours, glioblastoma, chronic lymphocytic leukemia and haemopoietic cancers among others (reviewed by Boggs et al., 2008). These could also be some of the reasons why our data show conflicting results with some of the studies mentioned above.

Furthermore, *Swarbrick et al, 2010* observed that human miR-380-5p regulates the expression of p53 in neuroblastoma, and thus, the inhibition of this miRNA increases p53 activity, inducing apoptosis. They found that high expression of miR-380 in neuroblastoma was correlated with poor prognosis in neuroblastoma, and after knockdown of miR-380-5p *in vitro*, they observed upregulation of p53 and consequently a higher apoptotic rate. Moreover, they transfected mouse mammary epithelial cells with miR-380-5p before transplantation into mammary fat pads of mice, and observed that miR-380-5p-transfected cells gave rise to a greater frequency in tumour formation than the control groups (Swarbrick et al., 2010). These results are in line with our data, where miR-380 was upregulated in TGF-β-stimulated canine mammary carcinoma cells *in vitro* (which, as described in Chapter 4.3, acquire resistant features associated with a more malignant phenotype). Although *Swarbrick et al., 2010* assessed miRNA expression changes in human neuroblastoma, which is a different type of cancer from a different species; this gives an idea for future research in this respect. We could perhaps assess if canine miR-380 also regulates the expression of p53 in canine mammary carcinoma cells and whether the inhibition of said miRNA enhances p53 activity, inducing apoptosis. An

interesting way to assess the correlation between TGF-β exposure and miRNA expression changes in canine mammary cancer cells would be to assess p53 activity in TGF-β-stimulated and unstimulated cells. This way we could confirm the regulatory activity of different miRNAs on p53 and correlate these changes with TGF-β-induced EMT and resistance to apoptosis.

There are no previous studies reporting a full screening of miRNA expression in canine mammary carcinoma cells associated with EMT-associated changes. Our data were validated by specific qRT-PCR assays targeting the mature miRNA sequences suggesting further research into the roles of miRNAs in other cancers and their inhibition is warranted. Different research groups investigated the role of specific miRNAs without global miRNA analysis in canine mammary carcinoma (Boggs et al., 2008, von Deetzen et al., 2014). *Boggs et al*, 2008 studied10 specific canine miRNAs based on previous human breast cancer miRNA expression profiles. Most miRNAs displayed similar expression profiles to those observed in human samples (Boggs et al., 2008). On a similar approach, *von Deetzen et al., 2014* assessed the expression of 16 specific miRNAs in canine mammary tumours and normal mammary gland samples. From these studied miRNAs, they observed the most significant changes between primary tumours and metastatic cells in 5 miRNAs; including miR-29b, miR-101, miR-125a, miR-143 and miR-145. Interestingly, none of the upregulated miRNAs in our study were included in their research. The data obtained by these research groups is very useful for the study of specific miRNAs associated with cancer progression (Boggs et al., 2008 and von Deetzen et al., 2014), nevertheless, our data is more comprehensive and might discover miRNAs that have greater significance in the canine rather than merely ones which are specifically measured based on human data. Therefore, our study could be considered a more comprehensive assessment as to whether the canine is a good model for human cancer miRNA research.

Using a different approach, *Rybicka et al., 2015* investigated miRNA expression profiles in canine mammary cancer stem-like cells expressing stem cell antigen 1, CD44 and EpCAM (stem cell markers) derived from 3 different cell lines. They observed 24 downregulated miRNAs in cancer stem-like cells compared to

differentiated tumour cells. From these miRNA profiles, cfa-miR-10b expression was downregulated in cancer stem-like cells; consistent with the findings of *Iorio et al.,* 2005 in breast cancer tissue samples compared to normal tissue in humans. Interestingly, *Rybicka et al., 2015* also found that cfa-miR-15a expression was upregulated and cfa-let-7f expression was downregulated in cancer stem-like cells, opposed to the findings of *Boggs et al., 2008* who observed downregulation of cfa-miR-15a in canine ductal carcinomas and upregulation of cfa-let-7f in tubular papillary carcinomas, respectively, compared to normal mammary tissues. These data also support the context-specific role for miRNAs, as reviewed by *Boggs et al., 2008.*

Moreover, *Rybicka et al., 2015* correlated miRNA expression profiles to specific target genes, according to KEGG and BioCarta databases, where they found that target genes of significantly downregulated miRNAs were involved in the TGF-β signalling pathway, among other pathways, and interestingly, one of the most important genes found to be overlapping with different pathways was TGFβR1. In future research we could investigate these specific miRNA expression profiles not only in cells that had undergone a TGF-β-induced EMT, but also in cells that had acquired stem cell-like characteristics after TGF-β stimulation to fully elucidate the role of these miRNAs in TGF-β-induced EMT and TGF-β-induced stemness.

It is evident that there is still much to be understood regarding the roles of miRNAs in cancer. We have shown that specific miRNAs are involved in TGF-β-induced EMT of a canine mammary carcinoma cell line *in vitro*. The discovery of new miRNAs and the understanding of action of known miRNAs could be very useful for cancer research in companion animals; and dogs and cats might have an important role as animal models for cancer research in humans. Inhibition of miRNAs might become a therapeutic option in the future, although outstanding questions regarding their function in healthy and diseased organisms need to be answered. Further investigation could lead to novel diagnostic and therapeutic tools for breast cancer and its progression in both companion animals and humans. On a similar approach, *Jin et al., 2014* found specific miRNA molecules involved in colorectal cancer in humans, and they emphasise their studies in the development of

therapies against specific clinical targets of EMT-associated miRNAs as they are regarded as important oncogenes and tumour suppressor genes (Jin et al., 2014). As stated by Liu et al (Liu et al., 2013), different diagnostic and prognostic protocols could be developed by simple blood tests to analyse circulating miRNA expression in the serum of patients.

Chapter 6: Final Discussion

Here, we have shown that there is an association between EMT and the acquisition of stem cell characteristics in mammary carcinoma cells of companion animals. These finding are in accordance with the literature which supports that induction of EMT in human breast cancer cells leads to an accumulation of cancer cells with stem cell characteristics (Morel et al., 2008, Mani et al., 2008, Chen et al., 2011) such as increased self-renewal potential; resistance to therapeutic agents; and enhanced migratory properties. Our results further highlight the benefits of developing new EMT/stem cell-based diagnostic and therapeutic protocols to be of benefit in the treatment of companion animal cancer patients and for advancement of veterinary medicine. Moreover, this work reinforces that dogs and cats are good animal models for the human disease regarding progression and metastasis of cancer (Vail and Macewen, 2000, and reviewed by Porrello et al., 2006, Boggs et al., 2008, Gama et al., 2008, Cassali et al., 2011 and Rybicka et al., 2015).

Both canine and feline mammary carcinoma cells go through an EMT process similar to human cells, which were used as a comparative control in this study. All cells exhibited a more mesenchymal phenotype after EMT was induced by TGF-β treatment, as shown in Chapter 3.3.1. These phenotypic changes were concurrent with changes in EMT-associated protein and gene expression levels as demonstrated in Chapters 3.3.2 and 3.3.3, respectively. Furthermore, after treatment with TGF-β, both feline and canine mammary carcinoma cells acquired increased motility *in vitro* (Chapter 3.3.4). Metastatic cells also require invasive features to pass through surrounding tissues and membranes in order to be able to reach lymphatic and blood vessels, and subsequently migrate to their new microenvironment. It is important to separate the terms migration and invasion in experimental biology. Migration is defined as the directed movement of cells on a 2D substrate without any kind of obstacle. On the other hand, invasion is defined as cell movement through a 3D extracellular matrix (ECM), which represents a true barrier for moving cells, as reviewed by *Kramer et al., 2013*. Invading cells would have to modify their shape and secrete proteolytic enzymes in order to dissolve the ECM and pass through it. Migrating cells may be capable of moving in 3D matrices but without destructive or

proteolytic features (reviewed by Kramer et al., 2013). In order to confirm whether our studied cells become more invasive after EMT, further investigation is needed, including invasion assays with different cell lines stimulated with different TGF-β concentrations and selected time points *in vitro*. As reviewed by *Kramer et al., 2013*, a comprehensive *in vitro* metastasis assay is practically impossible to achieve in the near future (Kramer et al., 2013). Nevertheless, there are different means to assess cell invasiveness. It is always recommended to calculate the invasive index, which represents the ratio of invasive cells to migratory cells in order to determine the cell line's relative invasiveness (Kramer et al., 2013). Also, *in vivo* invasion assays would be required to confirm the invasive characteristics of these cells in a living organism. Currently, in the author's lab, we are investigating the role of EMT *in vivo* in a chicken chorioallantoic membrane-based model for breast cancer. A different approach to study these characteristics *in vivo* would be to inoculate immunocompromised mice with labelled canine and feline mammary carcinoma cells into different organs and track their migratory and invasive capabilities thereafter. To reduce the use of experimental animals, an *in vitro* model that could accurately resemble cancer cell invasiveness *in vivo* is necessary. As an example, with or without TGF-β stimulation, canine mammary carcinoma cells could be in 3D ECM gels and their invasive traits could be measured after a period of incubation. This type of model has been previously utilised by *Dolznig et al., 2011*, where they assessed the invasiveness of different tumour-spheroid cells *in vitro*. They showed that an ovarian carcinoma cell line produced two populations, with non-invasive cells in compact spheres and invasive cells with cellular astral outgrowth into the collagen gel (Dolznig et al., 2011). If we could carry out a similar invasion assay, we could assess the acquired invasive features of canine mammary carcinoma cells after the acquisition of stem cell-like characteristics after TGF-β stimulation, and investigate the potential correlation between TGF-β-induced EMT, invasiveness and stemness.

As there is limited data published on EMT in canine cancer, especially in canine mammary cancer, and given the context-specific effects of some of the major factors involved, direct comparison with some previous human and murine work is difficult (reviewed by Yang and Weinberg, 2008, Thompson et al., 2005, and Tarin

et al., 2005). The disparity between phenotypic and transcriptional data in our study, shown in Chapter 3.3.3 could also be due to the complexity of the different TGF-β roles during EMT and cancer progression (Shipitsin et al., 2007, and reviewed by Blobe et al., 2000, Derynck et al., 2001 and Singh and Settleman, 2010). Interestingly, we also found that EMT-associated translational and transcriptional changes are not always expressed clearly in TGF-β-stimulated feline and canine mammary carcinoma cells, as described in Chapters 3.3.2 and 3.3.3, respectively. This finding could be in part due to loss of Smad4 during tumourigenesis by modulating the function of TGF-β, regulating its tumour suppressor activity and enhancing its oncogenic properties (Levy and Hill, 2005, Deckers et al., 2006, Piek et al., 1999, Valcourt et al., 2005). The Smad family is thought to be important for the acquisition of tumour suppression and tumour promotion features (reviewed by Derynck et al., 2001) and is a well-characterised effector signalling pathway initiated by TGF-β receptors (reviewed by Miyazono et al., 2000, Massagué and Wotton, 2000). Smad4 plays an important role in the acquisition of migratory and invasive characteristics in epithelial cells after TGF-β stimulation (Deckers et al., 2006, Piek et al., 1999, Valcourt et al., 2005). Nevertheless, *Levy and Hill, 2005* propose that Smad4 inhibition can be of importance during tumorigenesis by modulating TGF-β-induced response so that oncogenic and tumour promotion functions are retained but tumour suppressor activities are lost (Levy and Hill, 2005). Further investigation is necessary to clarify the role of Smad4 in our studied cell lines after TGF-β stimulation in order to understand its role during TGF-β-induced EMT *in vitro*. Therefore, elucidating the potential roles of Smad4 among other transcription factors and the cross-talk between different molecular pathways should lead to a more in-depth understanding of EMT-associated changes in canine mammary cancer. Even though there are some studies that correlate the loss of Smad4 to cancer progression in human pancreatic (Lüttges et al., 2001), colon (Mesker et al., 2009) and colorectal cancer (Ahn et al., 2011); and mice gastric cancer (Xu et al., 2000), none of these studies correlate their findings to EMT-associated changes in canine mammary cancer. It would be ideal to assess the expression of Smad4 at the posttranscriptional level in epithelial cells with and without TGF-β stimulation, especially contemporaneous with TGF-β-induced morphological changes.

Our data suggest that treatment could be directed towards EMT at early stages of tumourigenesis, targeting TGF-β and its downstream effectors to prevent cell migration, self-renewal and the acquisition of several other stem cell-like characteristics, including dysregulated cell growth and metastasis. Inhibition of TGF-β as a therapeutic approach against cancer has been studied by *Derynck et al,* 2001 who concluded that due to its context-specific roles, inhibiting this growth factor might induce tumour regression in advanced cancers, but might stimulate growth in quiescent tumours. Their suggestion was to inhibit TGF-β signalling in advanced metastatic cancer, but to augment it as a factor inhibiting progression in cancer at early stages. Moreover, they suggest that Smad6 and Smad7 may inhibit the functions of Smad4 after TGF-β induction, which could also serve as a potential therapeutic intervention to prevent migration and invasion in cancer cells (reviewed by *Derynck et al., 2001).*

Furthermore, EMT leads to the acquisition of stem cell characteristics, and tumours have previously been shown to be comprised of a heterogeneous mix of cells, of which a typically small percentage are CSC (reviewed by Reya et al., 2001) and are capable of driving tumour relapse and cancer progression due to their self-renewal properties and resistance to chemotherapy and radiotherapy (reviewed by Dean et al., 2005, Diehn and Clarke, 2006, Phillips et al., 2006, Pang et al., 2013). We carried out experiments with promising results confirming the association between the acquisition of migratory properties in mesenchymal-like cells and the acquisition of stem cell characteristics including self-renewal and resistance to apoptosis and chemotherapy after a TGF-β-induced EMT. These findings could lead to further research and a potential development of novel diagnostic and therapeutic techniques for breast cancer in companion animals and humans.

We showed that feline and canine mammary carcinoma cells acquire self-renewal and resistant characteristics after treatment with TGF-β, as they were capable of forming bigger and more spheres compared with the untreated cells in harsh culture conditions, as described in Chapter 4.3.1. These spheres are thought to be representative of CSCs (Morel et al., 2008, Mani et al., 2008 and Pang et al., 2011). Interestingly; these spheres were expressing a more mesenchymal phenotype

at the translational level, as they showed decreased protein expression of epithelial markers compared with adherent cells. Canine and feline TGF-β-stimulated mammary carcinoma cells also showed an approximate 3-fold increase in the number of cells expressing the putative CSC marker CD133. This is consistent with a study that showed that CD133+ cells from head and neck cancer had stem cell characteristics and acquired EMT-associated changes (Chen et al., 2011). Both sphere forming abilities and the expression of CD133 in TGF-β-stimulated cells, along with the protein expression of mesenchymal markers in spheres; indicate a correlation between EMT and the acquisition of CSC characteristics in canine and feline mammary carcinoma cells. Moreover, different research groups have found that there is a close link between the expression of this CSC marker and EMT *in vitro* and *in vivo* in different types of cancer, including metastatic pancreatic cancer (Ding et al., 2014 and Nomura et al., 2015) and lung carcinoma in humans (Koren et al., 2015). *Ding et al., 2014* showed that CD133+ human pancreatic cancer cells were able to produce metastatic tumours in immunodeficient mice, however, after CD133 knockdown with shRNA, cancer invasion and metastasis was suppressed *in vivo* while the EMT-related genes Slug and N-cadherin, along with the mesenchymal marker fibronectin were downregulated *in vitro*. These data confirm that CD133 plays a crucial role in invasion and metastasis of human pancreatic cancer *in vivo* and *in vitro* by upregulating the expression of EMT-related genes and the mesenchymal marker fibronectin (Ding et al., 2014). This correlation between CD133 and EMT induction in pancreatic cancer cells was confirmed by *Nomura et al., 2015 in vitro*. They demonstrated that a different human pancreatic cancer cell line became more invasive after CD133 overexpression *in vitro*. Moreover, these invasive CD133+ cells showed an increased expression of EMT transcription factors Snail and Zeb1, and mesenchymal markers vimentin and N-cadherin, confirming the cells go through an EMT process following CD133 overexpression (Nomura et al., 2015). Furthermore, *Koren et al., 2015* assessed the correlations between mRNA expression levels of EMT transcription factors (BMI1 and Twist) and CSC markers (CD133 and ALDH1A1) in primary tumours and whole blood samples from human patients with non-small-cell lung carcinoma and metastatic disease, respectively. They observed that there is a correlation between the mRNA expression of BMI1 and CD133 in

primary tumours and whole blood samples. This correlation was confirmed by immunohistochemical analysis where coexpression of BMI1 and CD133 was present in tumour samples (Koren et al., 2015). In order to confirm these findings in canine and feline mammary carcinoma, we could expand our investigation by assessing the expression of EMT markers in CD133+ cells, but more importantly after CD133 knockdown. Also, we could include invasion assays before and after CD133 overexpression and after subsequent CD133 knockdown in CD133+ cells.

Previously, CSCs have been shown to be inherently resistant to conventional chemotherapy and radiation therapy (Phillips et al., 2006 and Pang et al., 2013). Here we implemented a chemosensitivity assay, utilising two chemotherapeutic drugs commonly used for breast cancer in companion animals, doxorubicin and mitoxantrone. Feline mammary carcinoma cells did not show resistance to either of the drugs, with or without previous stimulation with TGF-β, compared to mock-treated cells. In contrast, TGF-β-stimulated canine mammary carcinoma cells showed a remarkable resistance to doxorubicin at increasing concentrations compared to the untreated cells. In canine mammary carcinoma cells, we expanded this experiment to establish a correlation between resistance to chemotherapy and the expression of the stem cell marker CD133 with and without TGF-β stimulation. We utilised CD133- untreated cells, CD133+ untreated cells, CD133- TGF-β-stimulated cells and CD133+ TGF-β-stimulated cells. When comparing the CD133+ untreated cells with the CD133- untreated cells, the former showed greater resistance to doxorubicin. Interestingly, the TGF-β-stimulated cells were all resistant to the drugs used regardless of their CD133 expression, indicating that there are resistance mechanisms within TGF-β-stimulated cells that are not associated with surface CD133. Moreover, we observed that the CD133+ cells demonstrated greater resistance to the drugs used compared to CD133- cells with or without previous TGF-β stimulation, which supports that CD133+ could be a marker for cancer cells with stem cell-like features, as shown by *Pang et al., 2013, Ding et al., 2014, Koren et al., 2015* and *Nomura et al., 2015*. The overall conclusion of this experiment is that TGF-β-stimulated canine carcinoma cells are comparable in chemotherapy resistance to the CD133+ cells, consistent with canine mammary carcinoma cells

acquiring stem cell characteristics through a TGF-β-induced EMT. It has been shown that TGF-β regulation of CD133 expression might be partially dependent on the Smad pathway (You et al., 2010). In order to elucidate if this pathway is associated with chemoresistance mechanisms, we could investigate if TGF-β stimulation decreases or increases CD133 expression and resistance to chemotherapeutic drugs in canine and feline mammary carcinoma cell lines after Smad knockdown. We could further assess if CD133 knockdown leads to a downregulation or upregulation in the expression of Smad in these cancer cell lines.

Further investigation is needed to assess if these findings are relevant *in vivo*. The confirmation of these results, regarding EMT-associated changes and the acquisition of stem cell characteristics *in vitro* could be followed by further experiments including antagonists of downstream effectors of TGF-β such as small interfering RNAs (siRNAs) to elucidate the druggable pathway involved, with a view to developing rational EMT-targeted treatments.

On a different note, miRNAs have important roles in physiological processes (Foubert, et al., 2010). Aberrant expression of miRNAs has been linked with different types of cancer in humans, including breast, lung, endometrial, gastrointestinal and prostate cancer (Calin et al., 2002, Volinia et al., 2006, Esquela-Kercher et al., 2008, Chin et al., 2008 and reviewed by Croce, 2009). Knowing that some genes related to cancer can be promoted or suppressed by different miRNAs as mentioned in Chapter 5.4, we screened miRNA expression in canine mammary carcinoma cells with and without TGF-β stimulation to investigate for changes in miRNA expression in cells before and after undergoing EMT. We compared the miRNA expression in cells with and without TGF-β stimulation at days 1, 10, 17 and 23, including samples in which TGF-β was withdrawn at day 19 and kept growing until day 23. Several miRNAs were upregulated in TGF-β-stimulated cells compared with the control groups. We observed 4 canine miRNAs that showed similar significant changes throughout the different time-points analysed herein. These miRNAs were upregulated in samples treated with TGF-β compared with untreated cells, but were downregulated in samples in which TGF-β was withdrawn, compared with the TGF-β-stimulated cells. This result shows that the TGF-β withdrawal

samples have a similar miRNA profile to untreated cells; meaning that they might undergo a mesenchymal to epithelial transition (MET) returning to a more epithelial phenotype profile after TGF-β is withdrawn.

The miRNAs expressing these similar significant changes were cfa-miR-411, 381, 410 and 380. These miRNAs were upregulated from day 1, showing a peak at day 10. It is unknown why they show a peak at day 10; a possible explanation would be that after 10 days of treatment with TGF-β, cells have acquired a mesenchymal phenotype and no longer require stimulation for an EMT induction after 10 days, when these miRNA expressions started to decrease. Another possible reason would be that the constant stimulation with TGF-β have resulted in downregulation of receptors and pathways leading to less profound miRNA expression changes after 10 days. However, we observed that TGF-β-stimulated cells acquired a mesenchymal morphology after 6 days of stimulation and preserved it until TGF-β was withdrawn. A feasible way to corroborate these ideas would be to perform miRNA expression analyses in TGF-β stimulated cells and TGF-β withdrawal cells at many different time points and at different concentrations of TGF-β, while observing morphological changes and evaluating the protein and mRNA expression levels of EMT markers and transcription factors at all time points. This approach would help us elucidate the role of TGF-β in miRNA expression after a TGF-β-induced EMT in canine mammary cancer cells.

Our view is that these findings could give significant insight into the regulation of EMT in canine cancer cells and identified several potential targets, however, several research groups have shown significant results regarding these 4 miRNAs and their association with various types of cancer in humans, which were not fully consistent with our findings. For instance, in contrast to what we observed in canine mammary carcinoma, miR-411 and miR-381 were downregulated in breast cancer in humans in 2 different studies carried out by *van Schooneveld et al., 2012* and *Liu et al., 2013*, respectively. *Van Schooneveld et al., 2012* showed that miR-411 was downregulated in localised and untreated metastatic breast cancer, compared to healthy tissue samples, while *Liu et al., 2013* showed that miR-381 was downregulated in non-metastatic infiltrating mammary carcinoma, compared to

healthy tissues. These opposing results suggest that these miRNAs may be bystanders and/or perhaps act in a tumour and/or context specific manner. An important approach to investigate these features would be to inhibit these miRNAs in canine and human mammary carcinoma cells during and after EMT induction and assess if their inhibition alters measurable aspects of EMT such as morphological changes, chemotherapy resistance, sphere forming ability, etc. This would provide a better idea of which miRNAs are essential for EMT and thus worthwhile targeting in future canine mammary cancer research and therapeutic trials, perhaps as a model for the human.

We are the first group to globally assess miRNA expression in canine breast cancer cells in association with EMT-associated changes. Different research groups have investigated the role of specific miRNAs in mammary carcinoma and lymphoma, among other types of cancer in humans and companion animals (Boggs et al., 2008, Uhl et al., 2011, von Deetzen et al., 2014, Rybicka et al., 2015 and Mohammad et al., 2015), giving a relevant insight into the functions of these miRNAs during cancer progression and prognosis in companion animals as described in Chapter 5.4, however, EMT was not a focus of their studies. We observed that the expression profiles of individual miRNAs in canine mammary carcinoma cells were in line with previous research on different types of cancer in humans (Swarbrick, et al., 2010), however, some of our findings were contrary to human cancer research (Snowdon et al., 2011, Van Schooneveld et al., 2012, Torres et al., 2013, and Liu et al., 2013). These discordant outcomes are not unexpected as some miRNAs can bind to several target mRNAs and thus, can effect both tumour suppressive and oncogenic functions (Boggs, et al., 2008). Examples of this can be seen with the human miR-17-5p, which has been classified as a tumour suppressor (Hossain, et al., 2006) but can also act as an oncogene (Iorio, et al., 2005) in mammary cancer. It is evident that there is still much to be understood in this field, nonetheless, the discovery of different miRNAs in cancer and its progression may be useful for the development of new therapeutic strategies to inhibit their functions. Our results bring light into the elucidation of further questioning regarding the role of specific miRNAs during EMT in mammary carcinoma of companion animals.

Elucidating different functions of miRNAs associated with cancer and its progression will undoubtedly advance cancer diagnostics and gene therapies in animals and humans in the future, providing important tools to create targeted therapies. These observations make clear that collaborations between veterinarians and researchers are particularly necessary to obtain the desired objectives to cure cancer and its progression.

Targeted therapies are becoming more popular than conventional chemotherapy among cancer treatment options, thus, miRNAs and antimiRNAs will perhaps contribute extensively to these kinds of selective therapies, as reviewed by *Croce, 2009*. Moreover, dogs bearing tumours such as mast cell tumours and gastrointestinal stromal tumours might benefit from the inclusion of tyrosine kinase (TK) inhibitors, as demonstrated by different research groups (Zemke et al., 2002, Frost et al., 2003, London et al., 2003 and Klopfleisch et al., 2012). The significance of TK in veterinary oncology has not been extensively studied with the exception perhaps of mast cell tumours; however, many studies have been conducted in human breast cancer (reviewed by Ranieri et al., 2014). A growth factor receptor on mast cells, CD117/c-Kit, is vital for their survival and proliferation (Tsai, et al., 1991) and plays an important role in angiogenesis in canine mast cell tumours (Klopfleisch et al., 2012), and in canine and human mammary carcinoma as reviewed by *Ranieri et al., 2013*. These observations could lead to important advances in veterinary oncology research regarding the development of targeted therapies against specific receptors of known importance in cancer progression and metastasis. The role of TKIs and miRNA inhibitors could be further researched in veterinary and comparative oncology.

In conclusion, the role of EMT applying the CSC theory to breast cancer of dogs and cats has not been previously studied. Further directions for our research include confirmation of our findings *in vivo* to more robustly investigate whether TGF-β induces EMT and confers migratory, invasive and other stem cell-like characteristics to mammary carcinoma cells and thus its relevance in patients rather than model systems. This would allow us to confirm whether breast cancer cells undergoing EMT gain resistance to apoptosis and chemotherapy, and have enhanced

migratory or invasive capabilities, thus contributing to progression and metastasis. As the role for currently available chemotherapy in small animal breast cancer has not been robustly proven, a more personalised approach based on druggable molecular targets might be needed for the discovery of novel therapeutic protocols for specific cancers. According to our findings, further investigation is necessary to assess if components of the TGF-β pathway and specific miRNAs can regulate EMT and prevent cancer progression and metastasis in mammary carcinoma of companion animals, confirming what has been observed by different research groups in several types of cancer in humans and laboratory animals (Derynck et al., 2001, reviewed by Croce, 2009).

To all those cancer patients out there…

“Now, this is not the end, it is not even the beginning of the end, but perhaps it is, the end of the beginning.”

Sir Winston Churchill

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Appendix 1: Published papers and collaborations

1) Canine Mammary Cancer Stem Cells are Radio- and Chemo-Resistant and Exhibit an Epithelial-Mesenchymal Transition Phenotype

2) Epithelial-mesenchymal transition as a fundamental mechanism underlying the cancer phenotype

Cancers **2011**, *3*, 1744-1762; doi:10.3390/cancers3021744

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cancers

ISSN 2072-6694

www.mdpi.com/journal/cancers

Article

Canine Mammary Cancer Stem Cells are Radio- and Chemo-Resistant and Exhibit an Epithelial-Mesenchymal Transition Phenotype

Lisa Y. Pang *, Alejandro Cervantes-Arias, Rod W. Else and David J. Argyle

Royal (Dick) School of Veterinary Studies and Roslin Institute, The University of Edinburgh, Easter Bush, Midlothian, EH25 9RG, UK; E-Mails: A.Cervantes-Arias@sms.ed.ac.uk (A.C.-A.); rod.else@ed.ac.uk (R.W.E.); david.argyle@ed.ac.uk (D.J.A.)

* Author to whom correspondence should be addressed; E-Mail: lisa.pang@ed.ac.uk; Tel.: +44-131-650-7684.

Received: 11 January 2011; in revised form: 25 January 2011 / Accepted: 22 March 2011 / Published: 30 March 2011

Abstract: Canine mammary carcinoma is the most common cancer among female dogs and is often fatal due to the development of distant metastases. In humans, solid tumors are made up of heterogeneous cell populations, which perform different roles in the tumor economy. A small subset of tumor cells can hold or acquire stem cell characteristics, enabling them to drive tumor growth, recurrence and metastasis. In veterinary medicine, the molecular drivers of canine mammary carcinoma are as yet undefined. Here we report that putative cancer stem cells (CSCs) can be isolated form a canine mammary carcinoma cell line, REM134. We show that these cells have an increased ability to form tumorspheres, a characteristic of stem cells, and that they express embryonic stem cell markers associated with pluripotency. Moreover, canine CSCs are relatively resistant to the cytotoxic effects of common chemotherapeutic drugs and ionizing radiation, indicating that failure of clinical therapy to eradicate canine mammary cancer may be due to the survival of CSCs. The epithelial to mesenchymal transition (EMT) has been associated with cancer invasion, metastasis, and the acquisition of stem cell characteristics. Our results show that canine CSCs predominantly express mesenchymal markers and are more invasive than parental cells, indicating that these cells have a mesenchymal phenotype. Furthermore, we show that canine mammary cancer cells can be induced to undergo EMT by TGFβ and that these cells have an increased ability to form tumorspheres. Our findings indicate that EMT

induction can enrich for cells with CSC properties, and provide further insight into canine CSC biology.

Keywords: canine; breast cancer; cancer stem cell; drug resistance; TGFβ; EMT

1. Introduction

Mammary tumors are the most common neoplasms that affect female dogs (*Canis familiaris*), constituting half of all tumors in female dogs and from these approximately half are considered malignant [1-3]. In both women and dogs, the incidence of mammary tumor development increases with age, rarely occurring before 25 and 5 years of age, respectively [4] and is hormone dependent [5]. Canine mammary carcinomas have epidemiologic, clinical, morphologic and prognostic features similar to those of human breast cancer and therefore represent a comparative model to understand the underlying molecular mechanisms of carcinogenesis in both species [4-6].

Recently, studies have identified subpopulations of cells within tumors that are responsible for tumor initiation, growth and metastasis; these cells have been termed cancer stem cells (CSC) [7,8]. CSCs have the capacity to self-renew, to initiate and maintain the tumor, and can produce heterogeneous lineages of cancer cells to compose the bulk of the tumor [9]. The cancer stem cell model therefore proposes that tumor development is akin to abnormal organogenesis [9]. In addition, CSCs are resistant to many current cancer treatments, including chemo- and radiation therapy [10-13]. Therefore conventional therapies, while killing the bulk of the tumor cells, ultimately fail because they do not eliminate the CSC population, which survives to regenerate the tumor. Further understanding the properties and mechanisms of CSCs is essential in the development of effective-anti-cancer therapies. In humans CSCs were first identified in acute myeloid leukemia [14], and more recently in melanomas [15,16], glioblastomas [17] and epithelial cancers [18-22]. In the canine model, we were the first to identify CSCs of a canine osteosarcoma cell line [23], and have subsequently isolated CSCs from a range of canine solid tumors including glioma, haemangiosarcoma and squamous cell carcinoma (data unpublished).

Recent evidence has suggested that tumor progression and metastasis is dependent upon aberrant activation of epithelial to mesenchymal transition (EMT) in cancer cells, resulting in the acquisition of invasive and metastatic properties [24]. Classically, EMT is an evolutionarily conserved developmental pathway involved in tissue morphogenesis, organ fibrosis and wound healing [25]. The hallmark of EMT is the loss of cell surface E-cadherin, which is associated with disassembly of adheren junctions, acquired motility and expression of mesenchymal markers including Vimentin and Fibronectin [26]. The EMT program is regulated by multiple transcription factors, including Twist, Snail and members of the ZFH family [27-29]. It is now known that EMT activation is also associated with the maintenance of stem cell properties, and *in vitro* it has been shown that emergence of CSCs occurs as a result of EMT [30-32].

In this study, we identified and characterized a subpopulation of putative CSCs from a canine mammary carcinoma cell line. Distinctive tumorsphere forming ability and expression of embryonic stem cell markers were identified in this subset and correlated with intrinsic resistance to DNA

damaging drugs and ionizing radiation. This subset of putative CSCs was predominantly mesenchymal in terms of marker expression and invasive capacity. In addition we show, for the first time in canine cancer cells, TGFβ induction of EMT and subsequent enrichment of cancer stem cells.

2. Material and Methods

2.1. Cell Culture and Tumorsphere Formation

Canine breast cancer derived REM134 cells (a kind gift from Prof. R.W. Else, The University of Edinburgh, UK) [33] were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum and 100 µg/mL streptomycin (Invitrogen, Paisley, UK). For anchorage independent culture, REM134 cells were plated as single cells in ultralow attachment 6-well plates (Corning, CA, USA) at low cell density (1.5×10^4 cells/mL). Cells were grown in serum-free conditional medium, which contained DMEM/F12 supplemented with progesterone (20 nM), putrescine (100 µM), sodium selenite (30 nM), transferring (25 µg/mL), insulin (20 µg/mL) (Sigma Biochemicals, Dorset, UK), human recombinant bFGF (10 ng/mL) and EGF (10 ng/mL) (Peprotech, NJ, USA). Additional growth factors (100 µg/mL) were added to the media every other day. All cell cultures were maintained at 37 °C in a humidified CO_2 incubator.

2.2. Tumorsphere Forming Efficiency

The sphere forming ability of TGFβ treated and untreated cells was determined by resuspending cells in serum-free conditional medium at a density of either 6000, 3000 or 1000 cells/well of 6-well low adherence plate (Corning, CA, USA). All experiments were conducted in triplicate. Plates were maintained at 37 °C in a humidified CO2 incubator and were maintained as before. After 7 days, the numbers of colonies were counted in 5 fields per well and representative views were photographed.

2.3. RNA Extraction and Reverse Transcription PCR Analysis

Total cellular RNA was extracted using RNeasy® kit (Qiagen, CA, USA) and RNA quality was determined by A_{260} measurement. Semi-quantitative RT-PCR analysis of mRNA expression of stem cell specific genes including *Oct4*, and *Nanog* was performed using HotStar *Taq* polymerase (Qiagen, CA, USA) and the following specific primers:

Oct4 sense 5'-CTCTGCAGCCAATCAACCACAA-3'
antisense 5'-GGAGAGGGGGATGAGAAGTACAAT-3'
Nanog sense 5'-CTATAGAGGAGAGCACAGTGAAG-3'
antisense 5'-GTTCGGATCTACTTTAGAGTGAGG-3'
β-Actin sense 5'-CATGTTTGAGACCTTCAACACCC-3'
antisense 5'-GCCATCTCTTGCTCGAAGTCCAG-3'

2.4. Irradiation and Drug Treatments of Cells

Cells were irradiated in culture media using a Faxitron® cabinetX-ray system, 43855D (Faxitron X-ray Corporation, IL, USA), at a central dose of 2 Gy/min. Cells were irradiated at the stated doses.

Cells were treated with Doxorubicin (Pfizer, Sandwich, UK) over the indicated range of concentrations. Cells were treated with 10 ng/mL Tgfβ(Peprotech, NJ, USA) for the indicated times.

2.5. Protein Detection

Cells were lysed in urea lysis buffer (7 M urea, 0.1 M DTT, 0.05% Triton X-100, 25 mM NaCl, 20 mM Hepes pH 7.5). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis (SDS PAGE), transferred to Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridized to an appropriate primary antibody and HRP-conjugated secondary antibody for subsequent detection by ECL. Primary antibodies against β-actin and Vimentin were purchased from Abcam (Cambridge, UK). Antibodies against β-catenin, E-Cadherin and Fibronectin were purchased from BD Biosciences (Oxford, UK). Anti-Twist (L-21) was purchased from Santa Cruz Biotechnology (CA, USA). Secondary antibodies were HRP-conjugated rabbit anti-mouse IgG and swine anti-rabbit IgG, were obtained from DakoCytomation (Glostrup, Denmark).

2.6. Cell Viability Assay

REM134 cells were seeded in quadruplet in opaque 96-well plates (Corning, CA, USA) at 500 cells /well. A serial dilution of doxorubicin was added to the appropriate cells the following day. Alternatively, cells were treated with different doses of ionizing radiation. Dose-response curves were generated 72 hours after exposure. Cytotoxicity was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, USA), which quantifies the number of viable cells in culture based on quantification of ATP present. Data was averaged and normalized against the average signal of untreated/vehicle control treated samples.

2.7. Colony Formation Assay

Tumorspheres and corresponding adherent parental cells were trypsinised into single cells and seeded at 500 cells/10 cm plate. The cells were irradiated at 0 Gy, 1 Gy, 2.5 Gy and 5 Gy whilst in suspension. Plates were incubated at 37 °C in humidified CO_2 incubator until colonies were visible. Growth media was changed once a week. The colonies were fixed by incubating with ice-cold methanol for 5 minutes at room temperature. Colonies were stained with Giemsa stain (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instruction, and counted. Each experimental condition was assayed in quadruplicate.

2.8. Invasion Assay

The invasive ability of cells was determined using the QCM™ collagen-based cell invasion assay kit (Millipore, MA, USA) was used according to the manufacturer's instructions. Cells were seeded into the upper inserts at 1×10^5 cells per insert in serum-free DMEM. Cells were incubated at 37 °C with 5% CO_2 for 48 hours. None invading cells were removed. Cells that migrated through the gel insert to the lower surface were stained and quantified by colorimetric measurement at 560 nm.

2.9. Wound-induced Migration Assay

REM134 cells (1×10^6) treated with either TGFβ (10 ng/mL) or a vehicle control were seeded in 100 mm culture plates and cultured to at least 95% confluence. Monolayer cells were washed with media and then scrapped with a plastic 200 µl pipette tip. Cells were then incubated at 37 °C with 5% CO_2. The "wounded" areas were photographed by phase contrast microscopy at 0, 4, 8, 24, 28, 32 and 48 hours after scraping. The relative migration distance was calculated by the following formula: Relative migration distance (%) = 100 (A-B)/A, where A is the width of the cell wound before incubation, and B is the width of the cell wound after incubation. Results are expressed as the mean ± standard deviation.

2.10. Statistical analysis

The results were presented as the mean ± SD. Data were analyzed using analysis of variance and a Student's t test. p values of <0.05 were considered significant.

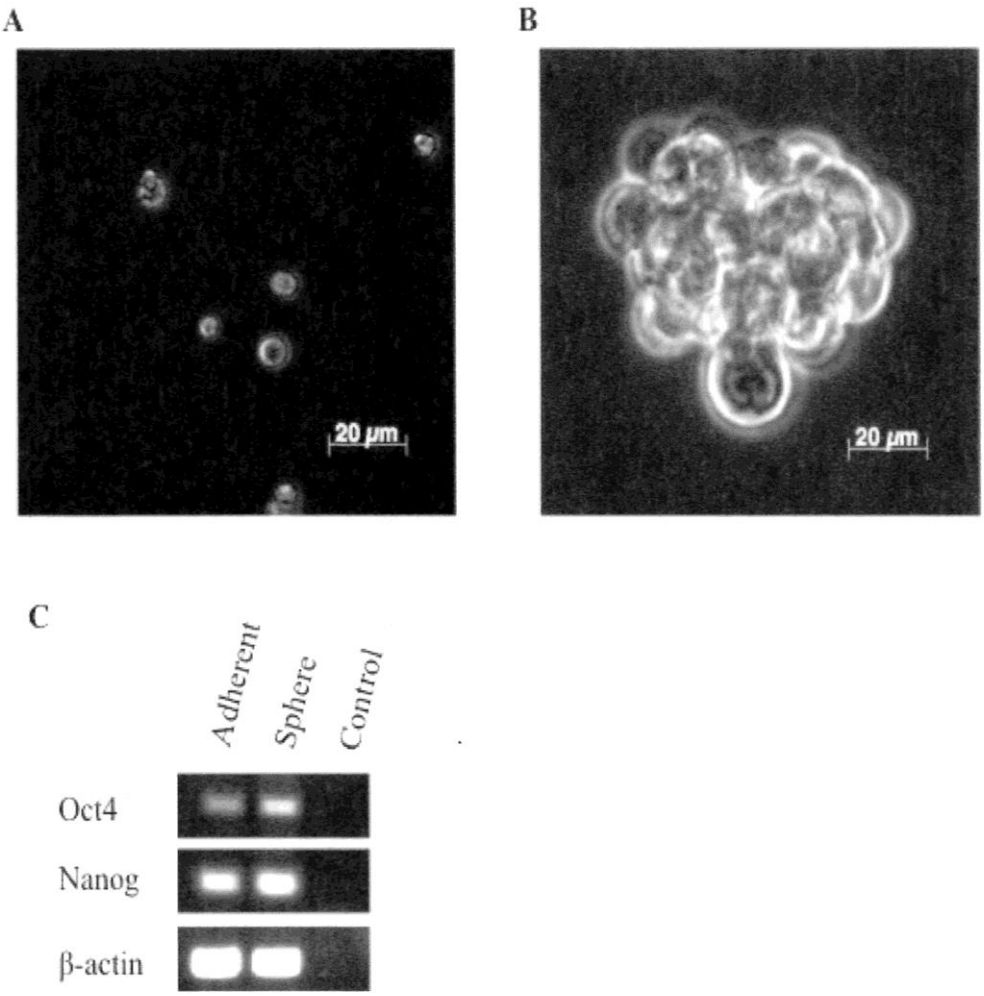
3. Results

3.1. A Subpopulation of Canine Mammary Carcinoma Cells Have Tumorsphere-forming Capacity

Previous studies have shown that cancer stem cells derived from a variety of human tumors tend to form spheroid colonies in defined serum free culture that favors the proliferation of undifferentiated cells [17,34,35]. Here, canine mammary carcinoma cells, REM134, were seeded as single cells at low-density into suspension cultures in serum-free growth factor supplemented media (Figure 1A). After 5–7 days tumorspheres were clearly visible (Figure 1B) and we estimated that approximately 1% of cells give rise to tumorspheres. To determine whether tumorspheres can be expanded *in vitro*, spheres were dissociated into single cell suspensions and passaged multiple times in long-term sphere forming assay. These cells repeatedly form tumorspheres for up to sixteen subsequent passages when plated under the stated culture conditions and in the absence of attachment.

To further characterize tumorspheres as a primitive sub-population of REM134 cells, we examined the expression of embryonic stem cell markers *Oct4* and *Nanog*. Oct4 and Nanog are transcriptional determinants essential for self-renewal and maintenance of the undifferentiated state [36]. Here we show that *Oct4* and *Nanog* are expressed at a higher level in tumorspheres compared to parental adherent cells (Figure 1C). Thus, the canine mammary carcinoma cell line, REM134, contains a sub-population of cells that can survive in the absence of attachment, forms tumorspheres that can be expanded *in vitro*, and express embryonic stem cell makers which may be required for maintaining these cells in a primitive state.

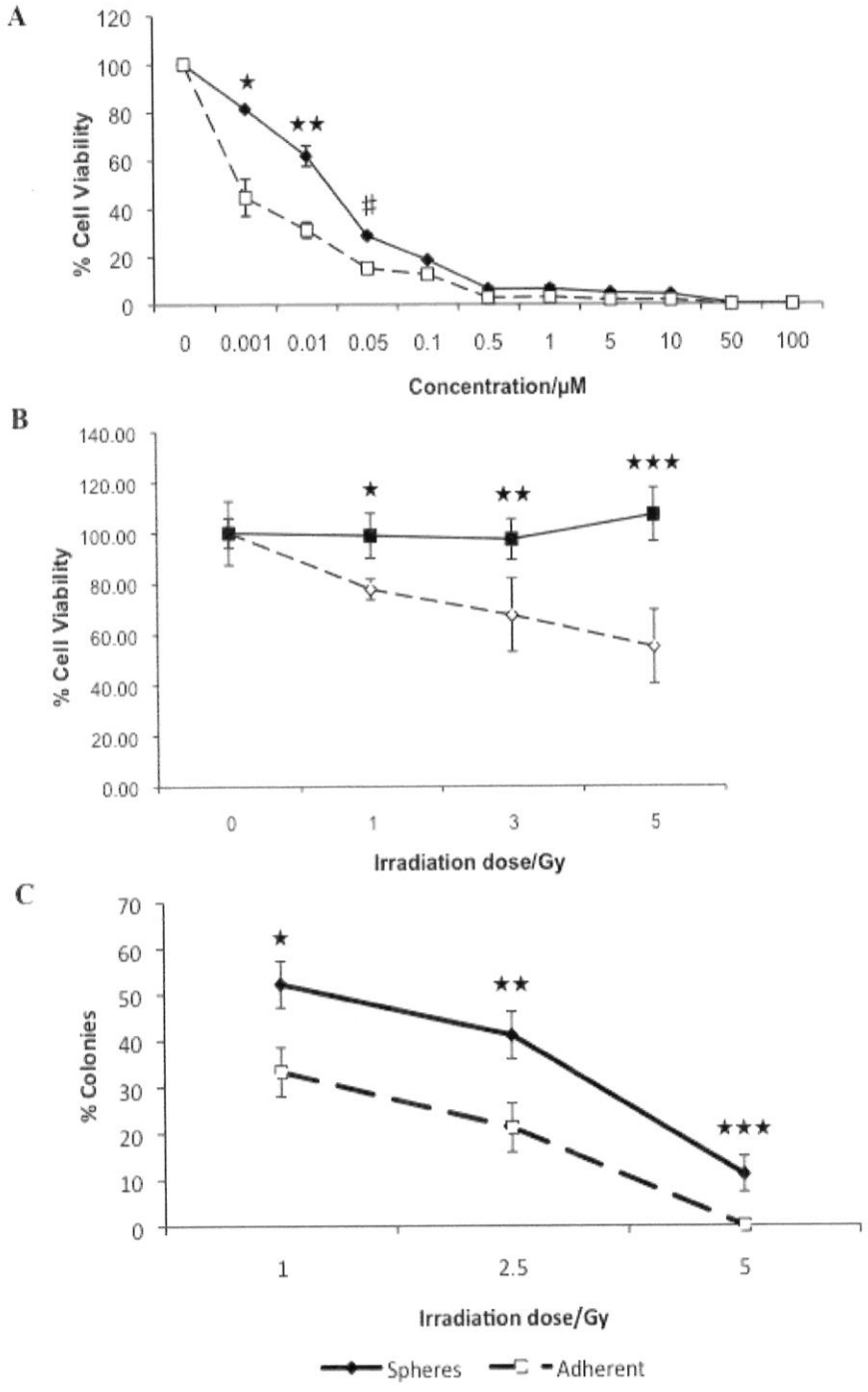
Figure 1. Isolation and characterization of putative cancer stem cells. Tumorsphere formation from the REM134 canine mammary carcinoma cell line. Single cells (A) and sphere (B). Non-quantitative RT-PCR analysis of mRNA expression of the embryonic stem cell markers *Oct4* and *Nanog* (C).



3.2. Canine Mammary Carcinoma Stem Cells Exhibit Greater Resistance to Chemo- and Radiation Therapy

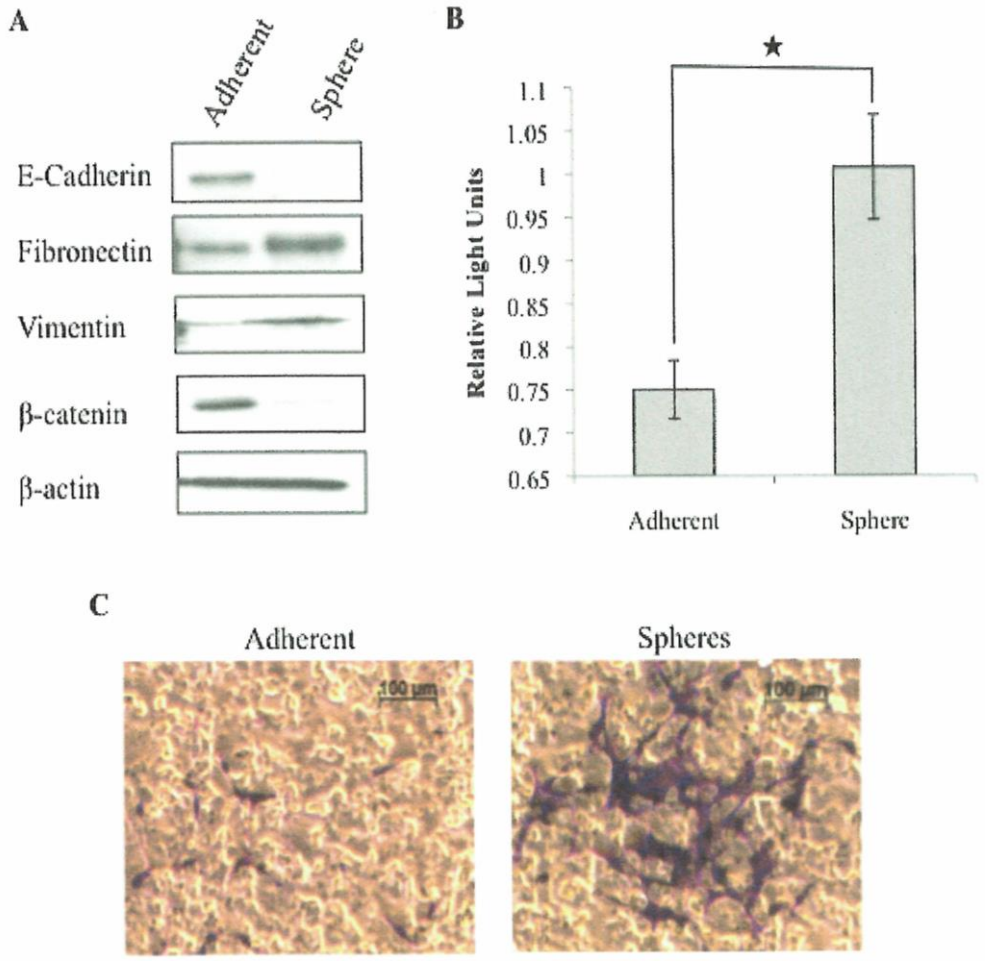
To determine whether tumorspheres cells preferentially survive after treatment with chemotherapeutic agents, tumorspheres were dissociated into single cells and treated with increasing concentrations of the cancer chemotherapeutic drug, doxorubicin. Doxorubicin is an anti-tumor antibiotic DNA damaging agent and is commonly used in veterinary and human cancer chemotherapy protocols. We used doses of Doxorubicin in cell culture experiments that correlate to concentrations that can be achieved *in vivo*. Cell viability was assayed 72 hours after treatment. Cells from tumorspheres demonstrated a significantly increased resistance to the cytotoxic effect of doxorubicin compared to parental adherent cells (Figure 2A).

Figure 2. Tumorspheres exhibit increased resistance to conventional chemo- and radiation therapies. Adherent cells and tumorspheres were treated with increasing concentrations of doxorubicin and cell viability was assayed 72 hours post-treatment (★ $p = 0.008$; ★★ $p = 0.038$; ♯$p < 0.001$) (A). Radiation sensitivity was determined by assaying for cell viability 72 hours post-treatment (★ $p = 0.003$; ★★ $p = 0.026$; ★★★ $p = 0.002$) (B) and by determining colony forming ability (★ $p = 0.01$; ★★ $p = 0.009$; ★★★ $p < 0.001$) (C).



Previously we have shown that tumorspheres derived from this cell line are more resistant to the anti-tumor effect of interferon-ω [37], consistent with increased resistance to doxorubicin. In addition, we compared the intrinsic radiosensitivity of cells dissociated from tumorspheres and parental adherent cells by measuring cell viability and clonogenic analysis. We used radiation doses based upon the therapeutic dose range. Adherent cells show a dose dependent decrease in cell viability, whereas tumorsphere viability is unaffected with increasing doses of ionizing radiation (Figure 2B). By colony formation assay we further confirmed that cells dissociated from tumorspheres are more resistant to ionizing radiation than corresponding adherent cells (Figure 2C). We have shown that REM134 cells with sphere-forming potential are more resistant to the therapeutic dose of DNA damaging agents and ionizing radiation *in vitro*, and therefore in a physiological setting may contribute to tumor repopulation.

Figure 3. Putative cancer stem cells exhibit mesenchymal characteristics. Tumorspheres derived from the REM134 canine mammary carcinoma cell line express mesenchymal markers (A). Invasive capacity was assayed using a collagen-based cell invasion assay kit. Invading cells are stained (C) and quantified by colorimetric measurement at 560 nm (□ $p < 0.008$) (B).



3.3. Tumorspheres Display Mesenchymal Features and are More Invasive

The metastatic process involves cell detachment from the extracellular matrix, migration from the tumor microenvironment and subsequent invasion and attachment at a secondary site within the body. Although the mechanisms underlying tumor cell invasion remain incompletely understood, EMT has been implicated by promoting loss of contact inhibition, increased cell motility and enhanced invasiveness [26,38]. Here we examined the expression of well-known epithelial markers (E-cadherin and β-catenin) and mesenchymal markers (Fibronectin and Vimentin) [39]. Tumorspheres were dissociated into single cells and compared to parental cells. Western blot analysis showed that the expression of E-cadherin and β-catenin was significantly decreased, whereas that of Fibronectin and Vimentin was significantly increased in tumorspheres compared to parental adherent cells (Figure 3A). Therefore tumorspheres that exhibit increased resistance to ionizing radiation and chemotherapy, have a mesenchymal phenotype.

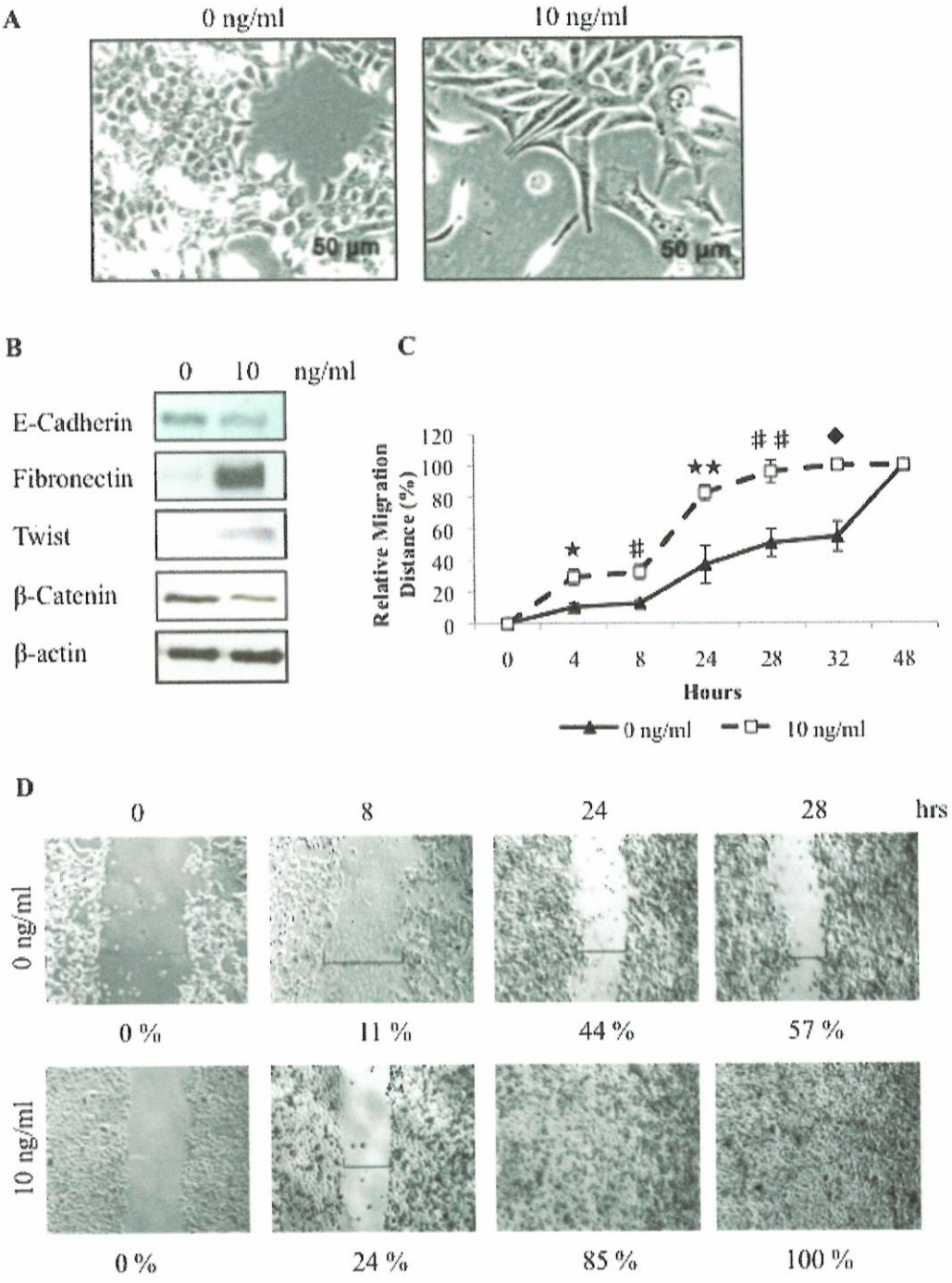
As EMT is associated with increased invasiveness, the invasive capacity of cells dissociated from tumorspheres and matched adherent cells was evaluated using the Boyden chamber assay. Tumorspheres displayed a significantly greater invasive potential compared to adherent cells (Figure 3B and 3C), consistent with the hypothesis that cancer stem cells contribute to invasion and migration of the tumor.

3.4 TGFβ Treatment of REM134 Cells Induces an Epithelial to Mesenchymal Transition and Enhances Tumorsphere Forming Potential

Previous studies have shown that EMT activation of human neoplastic mammary epithelial cells is associated with enrichment of cells with stem-like properties [31]. Here we have shown that canine tumorspheres have a mesenchymal phenotype and increased invasiveness, and may have undergone EMT. To investigate if an experimentally induced EMT in canine mammary carcinoma cells can also result in enrichment of putative cancer stem cells, we treated these cells with TGF*β*, a potent inducer of EMT. Within 72 hours of treatment with TGF*β*, the cells show a clearly manifested morphological change. The untreated cells are characterized by a cobblestone appearance whereas TGF*β* treated cells have an elongate fibroblastic phenotype indicative of mesenchymal cells (Figure 4A). The morphological change is associated with changes in protein expression of molecular markers of EMT. In the TGF*β* treated cells there is a decrease in the epithelial markers E-cadherin and β-catenin, and an up regulation of the mesenchymal markers Fibronectin and Twist (Figure 4B). This data supports the hypothesis that TGF*β* can activate EMT in canine cells.

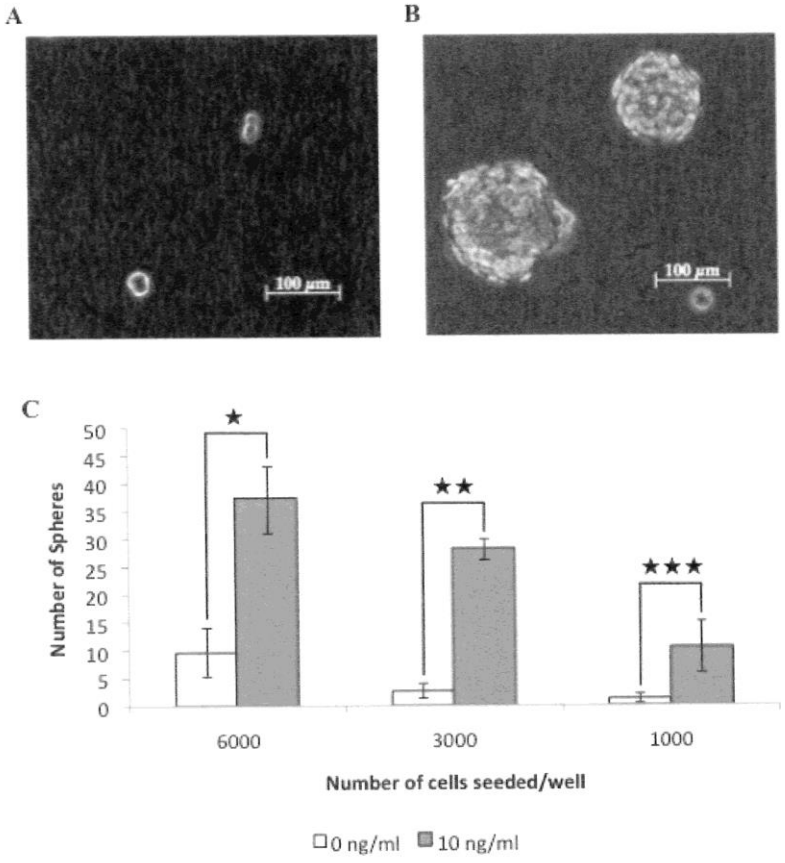
To investigate the effect of TGF*β* on cellular migration, monolayer wound-induced migration assays were performed. A wound was made in a sub-confluent cell monolayer and cells were allowed to migrate into the cell-free area. The distance moved by cells in the untreated and TGF*β* treated cells was compared. TGF*β* treatment significantly enhanced the migration and wound healing capacity of REM134 cells (100% closure of the wound in 28 hours) as compared to untreated cells (57% closure in 28 hours) (Figure 4C and D). Thus, the migratory potential of REM134 cells is enhanced by TGF*β* treatment.

Figure 4. Treatment of canine mammary carcinoma cells with TGFβ can induce an epithelial to mesenchymal transition, as indicated by changes in cell morphology (A), protein expression levels (B), and increased migration ability ($p = 0.018$; ♯$p = 0.014$; $p = 0.004$; ♯♯$p = 0.002$; u$p = 0.001$) (C, D).



EMT activation is proposed to enrich the proportion of cancer stem cells with a given cell population. Therefore we tested the ability of TGFβ treated cells to form tumorspheres when grown in suspension cultures, as an *in vitro* measure of cancer stem cell activity. TGFβ treated cells formed large clearly identifiable tumorspheres after 7 days in culture (Figure 5A) and showed an ~8-fold increase in tumorsphere forming ability relative to untreated cells (Figure 5B). This data indicates that canine mammary carcinoma cells induced to undergo an EMT by TGFβ contained a significantly greater proportion of cells with a CSC-like phenotype compared to control cells.

Figure 5. TGFβ treated cells show an increased tumorsphere forming ability compared to untreated cells. Tumorspheres resulted less frequently from untreated cells (A), compared to cells treated with 10 ng/ml TGFβ (B). The resultant number of spherical colonies were counted (□ $p < 0.001$; □□ $p < 0.001$; □□□ $p < 0.01$) (C).



4. Discussion

Breast cancer is a major cause of morbidity and mortality in women. However, the majority of cancer related therapeutic studies rely upon rodent models of human cancer that rarely translate into clinical success in human patients [9,40]. Recent advances in veterinary medicine, notably vaccination

regimes for once fatal infectious diseases, has led to an increase in age-related diseases of our pet dogs and cats which mirrors the pattern of human public health. In the UK, approximately 1 in 3 dogs will develop cancer, and with approximately 7 million dogs resident in the UK, this provides an exciting opportunity to exploit these cancers in terms of identifying cancer-associated genes, identifying environmental risk factors and understanding tumor progression [9]. In contrast to rodent models of human cancer, cancers that arise in dogs develop naturally and in the context of an intact immune system where tumor, microenvironment and host are syngeneic. Histologically, the majority of human cancers are well represented in the canine population, including breast cancer, melanoma, head and neck squamous cell carcinoma and osteosarcoma, and follow a similar clinical course [41]. Importantly, these observed similarities can be supported with genetic evidence, with the publication of the canine genome and the increased portfolio of molecular tools available for this species [42]. For example, a recently constructed syntenic karyotype map between humans and dogs demonstrated strong similarities in cytogenetic abnormalities in Non-Hodgkin lymphoma occurring in both these species [43].

With regards to breast cancer, the gene expression profile of metastatic canine mammary carcinomas has been determined by utilizing the canine specific affymetrix array. Importantly, this expression profile significantly overlaps with expression profiles of metastatic human breast cancer. In the subset of overlapping genes there is enrichment of genes associated with cell cycle regulation, protein kinases, DNA integrity checkpoint and protein metabolism [44]. In humans, several genes predisposing to breast cancer have been identified, but the majority of risk factors remain unknown. Even less is known about the inherited risk factors underlying canine mammary tumors. Germ line mutations in BRCA1 and BRCA2 account for 5% to 10% of all breast cancer in women [45], and correspondingly in dogs, germ line mutations in the same genes, as determined by candidate gene association, have also been shown to predispose to canine mammary carcinoma [46]. This recent evidence indicates that the molecular drivers and mechanisms of canine and human carcinogenesis are analogous. Here we further contribute to the evidence that canine mammary carcinoma is a model system that can be used alongside traditional rodent models to study the equivalent human disease. In the present study we utilized the REM134 cell line, and report that a subpopulation of canine mammary carcinoma cells may be representative of canine cancer stem cells. Putative CSCs were characterized by their tumorsphere forming capacity, expression of embryonic stem cell markers and resistance to chemo- and radiation therapy. These results are comparable to the human model of mammary carcinoma. In future studies we intend to evaluate the *in vivo* tumorigenic potential of isolated canine CSCs, and to investigate if CSCs can be isolated from primary canine breast cancer tissue.

Breast cancer was the first solid tumor from which CSCs were isolated, and this seminal paper provided compelling evidence for the presence of functional heterogeneity within the tumor population [18]. CSCs exhibit an ability to undergo self-renewal while sustaining a multipotent differentiation capacity to maintain tumor development indefinitely [7,47]. Previous studies have also shown that CSCs can be isolated from established cell lines, indicating that they are also maintained in a cellular hierarchy [48,49]. Human breast cancer stem cells were initially isolated by high expression of CD44 and low expression of CD24[18]. We have characterized a diverse range of established canine cancer cell lines, including REM134 mammary carcinoma, D17 osteosarcoma, J3T glioma, SB haemangiosarcoma, 3132 B-cell Non-Hodgkins lymphoma, and have determined that expression of

CD44 in canine cancer cells is cell cycle dependent (data unpublished) and is therefore not representative of a CSC marker in this species. However, sphere formation is also an established technique used to enrich stem cells [17], and here we show that a subpopulation of canine mammary carcinoma cells can promote clonogenic tumorsphere formation and be serially passaged for multiple generations. We further characterized tumorspheres using the embryonic stem cell markers, Nanog and Oct4. Constitutive expression of the transcription factor Nanog maintains the stem cell phenotype, allowing for self-renewal and propagation [50]. Similarly, Oct4 is a POU family transcription factor, which is initially expressed in the inner cell mass of the embryo and is essential for the maintenance of pluripotency [51]. We found that canine tumorspheres express higher levels of the *Oct4* and *Nanog* compared to parental cells. This data supports that tumorspheres have a primitive phenotype and are representative of a CSC population.

After surgery, ionizing radiation and chemotherapy are the most effective therapy for treating both human [52] and canine mammary carcinomas, however their effectiveness remains only palliative because of the ultimate development of treatment resistance [53,54]. The CSC theory proposes that CSCs are inherently resistant to conventional therapies and have the ability to repopulate the tumors after treatment [11]. Our results confirmed that tumorspheres derived from a canine mammary carcinoma cell line are more resistant to the chemotherapeutic drug doxorubicin and ionizing radiation, compared to parental cells. The molecular mechanisms underlying the intrinsic resistance of CSCs to conventional therapies remain elusive. However, previous studies have shown that CSCs express high levels of drug transporters [55,56]; anti-apoptotic proteins [57,58]; and preferentially activate DNA damage pathways[10]; Wnt/β-catenin pathways [59] and Akt/PKB survival pathways [60]. Interestingly, EMT of tumor cells has also been shown to contribute to drug resistance [25,30,61,62], although this remains mechanistically undefined.

Compelling evidence exists relating EMT to tumor metastasis and more recently to the emergence of CSCs. EMT is classically associated with the process of tissue morphogenesis during embryonic development, and is characterized by complex changes in gene expression [24]. Thus, epithelial and mesenchymal cells can be clearly distinguished by the expression of a number of markers [24]. EMT during embryonic development involves the loss of polarity and gain of motile characteristics of mesenchymal cells, which has prompted comparisons with metastatic cancer cells during malignant progression[63]. Subsequent studies have shown that EMT is a key step towards cancer metastasis, and that induction of EMT enhances cancer metastasis through enhanced invasion [64]. Here, we characterised tumorspheres and parental adherent cells in the context of EMT markers. We clearly show that tumorsphere cells have a mesenchymal phenotype, including down regulation of epithelial markers E-cadherin and β-catenin, and up regulation of mesenchymal markers Fibronectin and Vimentin, compared to parental adherent cells. Significantly, we also show that tumorspheres are more invasive than parental adherent cells, which is also indicative of a mesenchymal phenotype. Our findings are coherent with human breast cancers and breast cancer cell lines which display features of EMT [65,66], and with recent studies which have reported that loss of E-cadherin expression is positively correlated with advanced histological grade, metastasis and decreased survival [67,68].

Induction of EMT in normal or neoplastic mammary epithelial cells, by TGFβ treatment or siRNA-mediated inhibition of the human *CDH1* gene that encodes E-cadherin, has been shown to result in the enrichment of cells with stem-like properties [31,32]. This observation has enabled, for the first time,

high-throughput screening to identify compounds with specific activity against CSCs [30]. To our knowledge, we are the first to show that TGFβ treatment of canine mammary carcinoma cells can induce a change in cell morphology, expression of EMT markers and increased invasiveness consistent with induction of an EMT. Significantly, we also show that TGFβ treated canine mammary carcinoma cells have enhanced sphere-forming ability, indicating that EMT induction can enrich for canine cancer stem cells. CSC populations typically comprise only small minorities of cancer cell populations, typically less than 1% [69,70]. Our data confirms that canine CSC populations can be enriched *in vitro* by induction of EMT. This may enable us to further elucidate the mechanisms of CSC biology in the context of epigenetic regulation, drug and radiation resistance, and metastasis.

5. Conclusions

In summary, our data demonstrates that a putative cancer stem cell population can be isolated from a canine mammary carcinoma cell line. We show that these cells express embryonic stem cell markers, are invasive and are inherently resistant to radiation and chemotherapy. Significantly, we show that EMT induction can enrich the cancer stem cell population, and may be exploited to evaluate novel pathways to be targeted to increase therapeutic response in a clinical setting. Our results are consistent with human breast cancer models, and ultimately support the use of companion animals as a pre-clinical model system.

Acknowledgements

We thank Mrs. Rhona Muirhead for technical assistance. The authors' work is supported by grants from the Biotechnology and Biological Sciences Research Council (BBSRC) (LYP) and CONACYT (Mexican Government) (AC-A).

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Veterinary and Comparative Oncology

Review Article

DOI: 10.1111/j.1476-5829.2011.00313.x

Epithelial-mesenchymal transition as a fundamental mechanism underlying the cancer phenotype

A. Cervantes-Arias, L. Y. Pang and D. J. Argyle

Royal (Dick) School of Veterinary Studies and Roslin Institute, The University of Edinburgh, Easter Bush, Midlothian, UK

Abstract

Epithelial–mesenchymal transition (EMT) is a complex process involved in embryonic development, wound healing and carcinogenesis. During this process, epithelial cells lose their defining characteristics and acquire mesenchymal properties: loss of cell–cell adhesion; increased motility and invasiveness; resistance to apoptosis and changes in cellular morphology. EMT has been implicated as a driver of metastasis and tumour invasion, as this process allows cells to detach from their niche and migrate through blood and lymphatic vessels to invade different organs. This transition involves a diverse range of transcription factors, including Twist, Snail and ZEB1, and downstream transcriptional targets, including E-cadherin, β-catenin, fibronectin and vimentin. Recent evidence indicates that cancer stem cells are required for metastatic tumours to become established at a distant site, and that cancer cells undergoing EMT may develop stem-cell characteristics as well as increased invasive potential. The role of EMT in cancer biology is newly emerging in the human field, and to date very little has been done in veterinary medicine. EMT may therefore be an important molecular determinant of tumour metastasis, and further understanding of this process may lead to novel drug targets to be exploited in both veterinary and human medicine.

Keywords
cancer stem cell, cell reprogramming, comparative oncology, EMT, MET, metastasis, TGF-ß

Correspondence address:
A. Cervantes-Arias
Royal (Dick) School of Veterinary Studies and Roslin Institute The University of Edinburgh
Easter Bush, Midlothian
EH25 9RG, UK
e-mail:
A.Cervantes-Arias@sms.ed.ac.uk

The role of EMT in development

Epithelial–mesenchymal transition describes a rapid and often reversible change of cell phenotype from epithelial to mesenchymal,[1] and can facilitate cell migration. This program is essential throughout different embryonic stages such as organogenesis and gastrulation. Cells acquiring mesenchymal characteristics are able to move towards different places in the organism in order to generate organs and their anatomical structures.[2,3] This process is also proposed to play a role in metastasis by increasing cell motility and invasiveness. Based upon the fundamental roles that EMT has, the process has been classified as follows:

Type I Embryogenesis and organ development

Type II Fibrosis – wound healing and tissue regeneration

Type III Metastasis – enabling neoplastic cells to invade different tissues and organs during cancer progression.

EMT essentially mediates reorganisation of the cytoskeleton by decreasing cell–cell contact and changing cell polarity. Epithelial cells adhere to each other by lateral cell-cell junctions. These cells are polarized such that the bottom is defined as basal, and the top as apical. The matrix binding areas are located at the basal aspect of the epithelium. Due to these properties, epithelial cells generally form groups with tight junctions. In contrast, mesenchymal cells have a more elongated shape and their polarity is reversed, therefore they do not form large groups, and easily migrate (Fig. 1).[4] These changes are induced by transcriptional repression of proteins, such as cadherins, occludin, claudin and

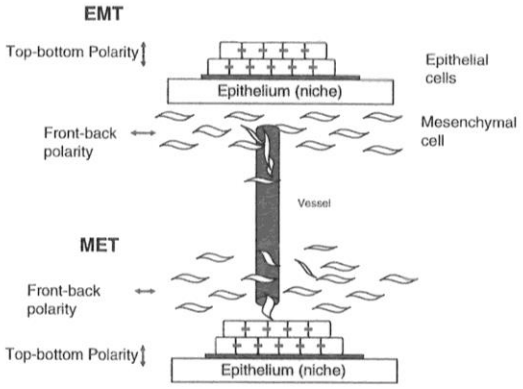

Figure 1. Polarity changes in cells undergoing EMT and the reverse program MET. Epithelial cells acquire front–back polarity and lose cell–cell contact, becoming mesenchymal and migrating through surrounding tissues and blood and lymphatic vessels. Once they reached the new microenvironment (niche), they regain their epithelial characteristics by MET.

desmoplakin. This repression can be accomplished by a range of transcription factors including ZEB1, Snail, Slug and Twist. Furthermore, epithelial cells undergo cytoskeletal changes, acquiring increased motility and invasiveness.[5,6]

If a cell is to achieve colonization of a distant site, then EMT has to be reversed. This reversion is called mesenchymal to epithelial transition (MET), and without it, cells would not be able to form any tissue or organ, including any kind of metastatic colonization. After MET, cells gain back their lost polarity and adhere to each other by tight junctions. This is due to an upregulation of proteins that help create adherens junctions such as E-cadherin and β-catenin, and the downregulation of those which can enable cells to break their adhesions and change their shape and polarity, such as fibronectin and vimentin. All three sub-types of EMT (types I–III) require the reverse MET program to enable cells to attach to their new microenvironments and establish a niche.[7]

EMT in cancer and during metastasis

Recent data shows that EMT is commonly observed in primary tumours at their cancer invasion front.[8] It is also associated with resistance to anticancer agents such as EGFR inhibitors.[9] According to the previously explained classification, during EMT III cancer cells follow similar pathways as normal cells during embryonic stages and wound healing. The existence of a reversible program (MET) which would enable cells to attach to a new epithelial sheet has been proposed by Thiery *et al.*[10] and Hugo *et al.*[11] Some of these cancer cells may also pass through just a partial EMT, expressing both, mesenchymal and epithelial markers during the process.[12]

There are three important pathways in EMT; Wnt/β-catenin, FGF and TGF-β1/BMP. These are vital for embryogenesis, but they also play important roles in tumour formation and progression. The transforming growth factor β1 (TGF-β1) pathway is crucial in EMT induction due to its multiple downstream effectors capable of repressing E-cadherin and subsequently enabling upregulation of mesenchymal promoters as fibronectin and vimentin.[13,14]

Signalling pathways and EMT inducers

To induce EMT during cancer, there are extrinsic and intrinsic stimuli. The microenvironment of a tumour plays a very important role as an extrinsic stimulus during cancer progression and metastasis. Some EMT inducing factors can be promoted by the interaction between the microenvironment and tumour cells, for example, members of the Snail family (E-cadherin repressors) can be activated by extracellular factors. Extrinsic stimuli include activators of Wnt, Hedgehog, Notch, nuclear factor-κB (NF-κB) and TGF-β signalling pathways. Subsequently, TGF-β is a potent EMT inducer during tumour progression.[9,15,16] Crosstalk between these signalling pathways has been mplicated in E-cadherin repression and EMT induction. For example, Wnt and Hedgehog signalling cascades induce Snail upregulation, which subsequently represses E-cadherin. Hedgehog signals interact with Notch and TGF-β signalling pathways to induce EMT.[17] Wnt signalling pathway has been shown to be disrupted during different cancer stages leading to EMT induction.

TGF-β is a member of a very complex multifunctional family of cytokines which act as mediators of development stages and tissue homeostasis. However, they have paradoxical

functions depending on cell type. TGF-β can act as a physiological and pathophysiological regulator through different stages of life.[18,19] For example; it can promote apoptosis and/or cell cycle arrest in normal epithelial, endothelial and haematopoietic cells, but when there is a deregulation or a mutation in the TGF-β pathway, apoptosis can be inhibited while cells acquire tumorigenic characteristics and also become motile and invasive to the surrounding tissues.[20] Mutations in the TGF-β type II receptor were demonstrated by Markowitz *et al.*[21] and Myeroff *et al.*[22] in different kinds of cancer showing loss of expression of this receptor. This resistance to its anti-proliferative characteristics can contribute to the formation of different kinds of cancer. The PI3K signalling pathway (induced by TGF-β), via its downstream effector Akt, can block apoptosis in mammary epithelial cells chronically treated with TGF-β by sequestering Smad3 into the cytoplasm after insulin treatment.[23,24] Moreover, TGF-β acts as a tumour suppressor during early stages of tumourigenesis by its growth inhibitory effects and stimulation of apoptosis. Interestingly, during late stages of tumour progression, it can also act as a tumour promoter with prometastatic effects. This was confirmed by Shipitsin *et al.*,[25] who observed a high level of expression of TGF-β in $CD44^+/CD24^-$ metastatic breast cancer stem cells (CSCs). In addition, following TGF-β inhibition, these cells demonstrated a more epithelial behaviour. This cytokine can stimulate two of the most important features of cancer progression, which are invasion and metastasis.[26] Furthermore, when TGF-β binds to its receptors (TGF-βRI and TGF-βRII), a signalling cascade is initiated by their phosphorylation, which can activate different proteins, in which the Smad family of transcription factors is one of the most notable. Specifically, when Smad2 and Smad3 bind with co-smad (Smad4) after their activation via TGF-βRI, this complexes associate with different transcription factors like ZEB proteins to repress E-cadherin during EMT.[19,27,28]

TGF-β also activates a range of downstream effectors, which can induce EMT. One of the most important downstream effector is NF-κB. As yet, the exact way NF-κB works is not fully understood, but one of its major roles is to suppress apoptosis. It remains unclear how NF-κB regulates EMT and metastasis, however, Huber *et al.*[29] demonstrated that EMT can be induced in Ras-transformed mammary epithelial cells by gain of NF-κB. They also demonstrated that inactivation of NF-κB causes MET, suggesting that this signalling pathway not only induces EMT but also helps to maintain it. Inhibitory proteins known as Inhibitor κB (IκB) proteins are involved in NF-κB activity regulation.[29] And by using these, NF-κB function has been confirmed in different model systems. Huang *et al.* used a mutant form of IκBα to downregulate NF-κB activity in metastatic human prostate cancer and, as a result, they observed inhibition of tumour growth, invasion and metastasis *in vitro* and *in vivo*.[29,30] Furthermore, they also confirmed this in human melanoma cells.[29,31] NF-κB regulates Snail expression, leading to its increased transcription. Subsequently, Snail represses Raf kinase inhibitor (RKI), which can inhibit NF-κB activity. Therefore, Snail overexpression suppresses E-cadherin activity, inducing EMT while repressing RKI.[32]

Genetic alterations such as mutations/perturbations can act as intrinsic stimuli of EMT during cancer progression by potentiating EMT inducers or inhibiting EMT repressors. TGF-β can act as a tumour suppressor after acquiring a specific gene mutation, but its unmutated form can function as an EMT inducer to promote invasion and metastasis.[27] Lehmann *et al.*[33] showed that during cancer progression, the TGF-β growth inhibitory effect can have a negative feedback and cancer cells can proliferate due to an upregulation of Ras proteins and consequential activation of the MAP kinase pathway. The same authors demonstrated that TGF-β lost its pro-apoptotic but not its pro-invasive activities through this pathway. TGF-β activates a range of different signalling pathways leading to EMT during tumour progression (Fig. 2), such as Rho family of GTPases (Fig. 2A), PI3K/AKT (Fig. 2B), integrin-linked kinase (ILK) (Fig. 2C), NF-κB (Fig. 2D) and MAP kinases (Fig. 2E).[26] Each of these signalling pathways involves different features that are inter-related, for example, downregulation of EMT suppressors such as E-cadherin or β-catenin by a

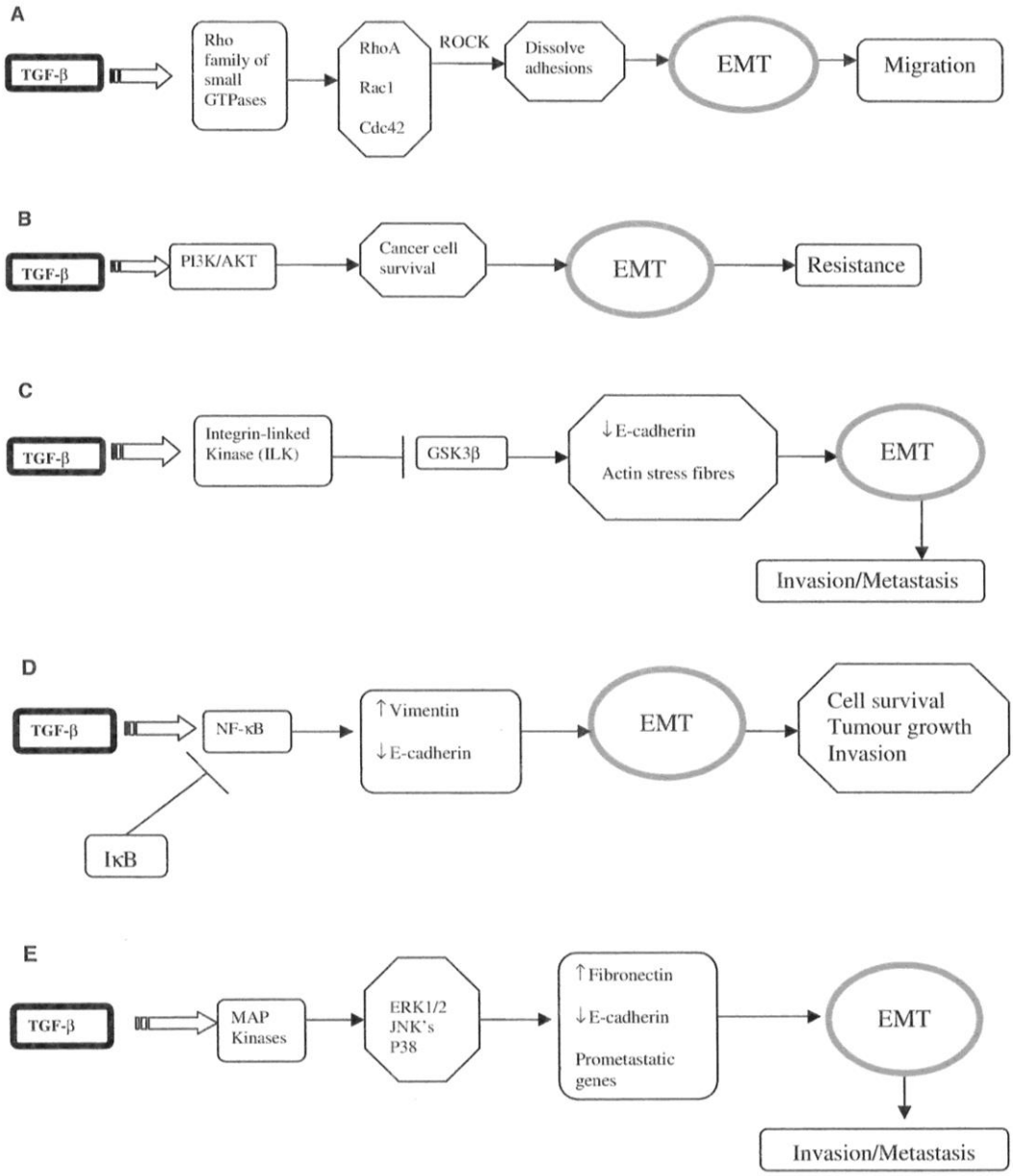

Figure 2. TGF-β1 stimulated signalling pathways. Among the signalling pathways stimulated by TGF-β1, there are at least these five that can promote EMT through different molecular changes. (A) Rho family of small GTPases is comprised by RhoA, Rac1 and Cdc42. If RhoA is activated via TGF-β, epithelial cells can acquire mesenchymal characteristics by losing their cell–cell adhesion and gaining motility in order to migrate. This can be achieved by RhoA and its downstream effector ROCK. (B) PI3K/AKT. This signalling pathway is crucial to achieve EMT because it enhances cancer cell survival. As we understand, when cells migrate through different tissues and vessels, they can be seriously damaged and killed. (C) Integrin-linked kinase (ILK). Its elevated expression can be associated with downregulation of E-cadherin (cell–cell adhesions) and with formation of stress fibres and invasion which can be due to upregulation of fibronectin and vimentin. ILK can also be coupled with the AKT pathway. (D) NF-κB. This pathway can stimulate cell survival by regulating apoptosis, tumour cell growth and immune response. TGF-β suppresses its activity in normal cells, but it can stimulate it in cancer cells, leading to EMT by downregulation of E-cadherin and upregulation of vimentin. NF-κB can be repressed by IκB to block EMT and prevent migration. (E) MAP kinases. This is a big family of protein kinases including ERK1/2, JNK's and p38 MAPKs. Activation of these family members by TGF-β promotes EMT by downregulating E-cadherin and increasing the formation of stress fibres with the upregulation of fibronectin. Invasion can also be achieved by the activation of prometastatic genes via p38 MAPKs.

 Veterinary and Comparative Oncology, doi: 10.1111/j.1476-5829.2011.00313.x

range of transcription factors including Snail, Slug, Twist, and ZEB1.[26]

Molecular features of EMT during tumour progression

There are four important stages in cancer development and cell migration which are (1) invasion, (2) intravasation, 3) extravasation and 4) metastatic colonization.[34] In order to complete each step during this process molecular changes are required to enable EMT. These molecular and genetic alterations are due to different transcription factors, which act as EMT inducers by stimulating and/or suppressing functions of different proteins involved in this complex process. Important transcription factors are Snail, Slug (Snail 2), Twist and ZEB1, they act in different ways, but all of them repress E-cadherin expression which is classified as a hallmark of EMT.

One of the principal characteristics of EMT is the loss of E-cadherin expression. E-cadherin participates in cell–cell adhesion and interacts with other molecules to form epithelial junctions. It connects epithelial cells by calcium-dependent homotypic interactions.[35] Its expression is inversely proportional to the tumour grade and stage, and patient prognosis. β-catenin is also essential for keeping cells attached to each other, it acts by binding E-cadherin and α-catenin to the actin cytoskeleton.[35–37]

Once epithelial cells detach from other cells and their epithelial sheet, they can acquire motility by changing their shape and polarity in order to migrate through different tissues and lymphatic and blood vessels. Vimentin is an intermediate filament protein with structural features which plays a main role in switching a cell's shape and making their cytoskeleton stronger so it can be more flexible and prevent damage. It binds with microtubules and actin microfilaments to make up the cytoskeleton. Without this protein, migrating cells would be very fragile. Not only is a robust cytoskeleton necessary for migrating cells, it is also necessary to guide these cells during migration. Fibronectin is a high molecular weight extracellular matrix glycoprotein which plays an important role in cell adhesion, growth, migration and differentiation.[38] It plays an important role by guiding cells and binding to collagen and fibrin to enable embryogenesis and wound healing. Genetic alterations in fibronectin have been associated with some pathologies like fibrosis and metastasis.[38–40]

The vast majority of molecular changes in these proteins are due to genetic alterations in different transcription factors which can be divided into different families. The Snail family is made up of Snail and Slug (Snail 2) and they are involved in different stages of development such as gastrulation, mesoderm formation, cell differentiation, cell motility and apoptosis.[41–44] They can be implicated in EMT by downregulating the expression of E-cadherin.[45–47] Olmeda *et al.*[48] showed that when Snail and Slug were inhibited in mammary tumour cell lines, tumour growth was impaired and their metastatic potential was lowered in mice. Twist is part of the basic helix-loop-helix protein family and one of its most important features is the ability to inhibit apoptosis. It is also known to trigger EMT by downregulating E-cadherin expression.[49,50] Two members of the zinc finger E-box binding proteins family are ZEB1 and ZEB2, both of which can regulate E-cadherin and thus, are capable of stimulating EMT (Fig. 3).[51–53] Burk *et al.*[54] showed that ZEB1 was overexpressed in colorectal, pancreatic and breast cancer cell lines undergoing EMT induced by TGF-β1 and TNFα. Moreover, when they knocked down ZEB1, EMT was partially prevented. Each of these different epithelial/mesenchymal biomarkers and transcription factors has different characteristics and functions as shown in Table 1.

Cancer stem cell theory

Recent studies have shown that a small population of cells within a tumour can have stem-cell characteristics, such as self-renewal and pluripotency. These cells have been named CSCs or tumour-initiating cells,[56] and are required to produce all cancer cell types in a tumour.[57] The concept that tumours can grow from small populations of tumorigenic cells was proposed in the late nineteenth century, but was tested by Dick et al.[58–62] approximately 100 years later, in 1994 with haematopoietic stem cells and leukaemia stem

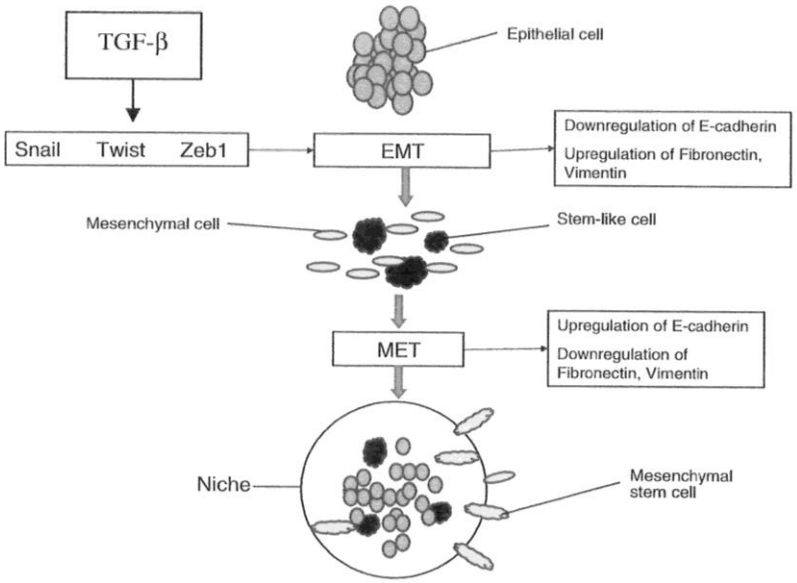

Figure 3. EMT induces cells to acquire stem-cell-like characteristics. After undergoing EMT, epithelial cancer cells acquire not only mesenchymal characteristics like spindle shape and front-back polarity, but also stem-cell-like features such as tumour-seeding abilities and resistance to apoptosis. However, once they reach their new microenvironment (niche), they undergo mesenchymal–epithelial transition (MET) in order to be capable of colonizing it.

cells. This seminal study, helped to define cancer stem cells, i.e., a single cell capable of generating a population of heterogeneous cells.[58–62] They confirmed that acute myelogenous leukaemia could be seen as a hierarchical model which emerged from a single haematopoietic stem cell by looking for expression of cell-surface markers. They found out that leukaemia-initiating cells that could form large number of colonies in transplanted severe combined immune deficient (SCID) mice were $CD34^+$ $CD38^-$. And cells that were $CD34^+$ $CD38^+$ and $CD34^-$ did not have the same colony-forming properties.[63] Different studies of neoplastic tissues are providing strong evidence of the existence of tumour-initiating cells.[56] These cells were first discovered in the haematopoietic system, but have subsequently been identified in several kinds of solid tumours including colon, breast and brain cancer.[64–67]

Linking EMT and cancer stem cells

Recent experiments have demonstrated that cancer cells induced to undergo EMT, produce cells with stem-cell characteristics.[68] In addition to morphological changes, epithelial cells induced to undergo EMT may also develop altered functional properties, such as tumour-seeding ability, tumoursphere formation and expression of transcription factors Twist and Snail. In addition, they also play an important role in invasiveness and migration in different types of cancer (Fig. 3).[69] Independent groups have demonstrated that cells that had undergone EMT by expressing different transcription factors or induced by TGF-β, showed stem-cell characteristics, such as the ability to form spheres and expressed stem cell associated cell-surface markers.[68,70,71]

Mani *et al.*[68] showed that by over expressing Twist or Snail in human mammary epithelial cells (HMECs), these cells became more mesenchymal, and their expression pattern demonstrated down-regulation of epithelial markers, such as E-cadherin, and upregulation of mesenchymal markers such as vimentin and fibronectin. After confirming that EMT took place in these cells, they used flow cytometric analysis to see if they had stem-cell characteristics. They confirmed that these cells expressed $CD44^+CD24^-$, which are cell-surface markers for mammary epithelial stem cells and human breast cancer stem cells. They achieved similar results exposing epithelial cells to TGF-β.[68] Furthermore,

Table 1. EMT biomarkers (epithelial and mesenchymal) and transcription factors characteristics as well as their expression during EMT

Protein/transcription factor	Characteristics	Expression during EMT	References
E-cadherin Epithelial	Cell–cell adhesion. Tumour suppressor gene	Downregulation	35, 46, 50, 51
β-catenin Epithelial	Adherens junctions. Transmits the contact inhibition signal once the epithelial sheet is complete	Downregulation	35, 36
Fibronectin Mesenchymal	Binds extracellular matrix components such as collagen and fibrin. Growth, migration, differentiation, wound healing. Cancer and fibrosis	Upregulation	13, 50, 55
Vimentin Mesenchymal	Make up the cytoskeleton. Supports the position of organelles in cytosol. Maintains cell shape and offers flexibility to the cell. Strengthens the cytoskeleton	Upregulation	13, 50, 54, 55
Snail	Critical for mesodermal development E-cadherin repressor	Upregulation	46, 47
Slug	E-cadherin repressor	Upregulation	46, 47
Twist	Cell lineage determination and differentiation	Upregulation	49, 50
ZEB1	Transcriptional repressor of microRNA-200 family members and EMT inducer	Upregulation	51, 52, 54

Every component of this complex network has different characteristics; some of which would enable cells to acquire mesenchymal features whilst losing epithelial properties. Their expression can be downregulated or upregulated, depending on their role in EMT.

they measured the sphere forming ability of normal mammary epithelial cells, compared with EMT induced cells with TGF-β and found that EMT induced cells were capable of forming at least 30-fold more mammospheres than the untreated cells. Moreover, they performed cell sorting in HMECs, mouse mammary stem cells and normal and neoplastic human breast stem cells and confirmed using RT-PCR the overexpression of mRNA's encoding mesenchymal markers and downregulation of epithelial markers in $CD44^+CD24^-$ (human) and CD49fhigh CD24med (mouse). They also transfected immortalized HMECs, transformed by the *HER2/neu* oncogene, with a vector expressing the tamoxifen-activatable form of Snail or Twist transcription factors in order to confirm the possibility of EMT generating cells with stem-like properties. These treated cells were assayed for tumoursphere forming efficiency. They found that cells expressing Snail and/or Twist underwent EMT, and formed 10-fold tumourspheres than the untreated group (control).[68] Further, they showed that EMT-derived cells were capable of differentiating into other cell lineages.[72]

Additional studies have confirmed that tumour-initiating cells can originate from a more differentiated cell line. Morel *et al.*[70] confirmed this by utilizing FACS analysis to demonstrate that cells expressing $CD44^+CD24^-$/low can be derived from cells expressing $CD44lowCD24^+$ through the activation of the Ras/MAPK signalling pathway, and most interestingly, that this process can be stimulated and accelerated by EMT. They compared the abilities of HMEC's and an oncogenic line (HMLER) to form mammospheres, a process associated with stem-cell properties. They confirmed that only HMLER cells were able to form mammospheres, and subsequently analyzed the cell phenotypes of these different cell lines by FACS confirming that HMEC's were $CD44lowCD24^+$ and HMLER were $CD44^+CD24^-$/low. Furthermore, they performed cell sorting and single-cell cloning assays after H-RasV12 retroviral expression in HMECs. They seeded $CD24^+$ cells into 96-well plates with limiting cloning conditions and saw that after three weeks, 19% of the population of cells were $CD44^+CD24^-$. This population of cells was able to grow tumours when injected

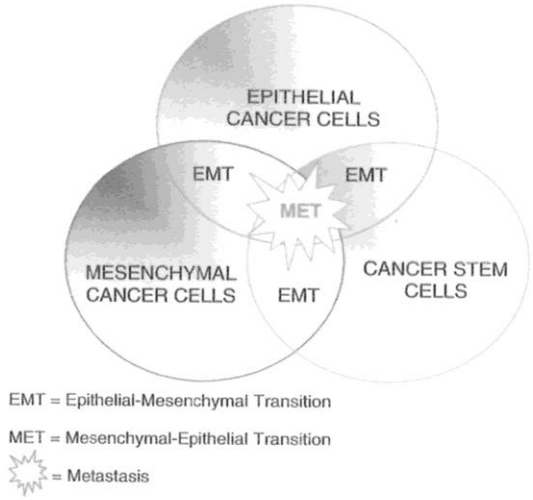

Figure 4. Crosslink between EMT and cancer stem cells lead to metastasis. Epithelial cancer cells acquire invasive and resistant phenotypes through an EMT and a cancer stem-cell characterization leading to metastatic progression and colonization of the new niche after the reverse program MET.

into mammary pads of nude mice, compared with CD44lowCD24$^+$, which were not able to establish tumour growth. CD44$^+$CD24$^-$ cells in HMEC and MCF10 (immortal human mammary epithelial cell line) cell lines showed a spindle shape and expressed lower levels of E-cadherin and β-catenin (epithelial markers) and higher levels of fibronectin and vimentin (mesenchymal markers), suggesting that these stem-cell properties were related to EMT. Finally, they confirmed that EMT enabled cells to acquire stemness by treating the CD44$^-$CD24$^+$ cell lines with TGF-β1, which is one of the most potent EMT inducers, for eight days and showed upregulation of vimentin and downregulation of E-cadherin, as well as presence of CD44$^+$CD24$^-$ cells.[70] Collectively, these results strongly suggest that EMT is potentially a precursor to generate cancer stem cells from more differentiated cell lines (Fig. 4). Further work is required to consolidate these theories.

Clinical and therapeutic implications of an EMT/CSC axis

Recent data demonstrates that epithelial cells induced by EMT may play an important role in invasiveness and migration in different types of cancer (Fig. 3).[69] The percentage of cancer stem cells in a tumour may vary between different tumours and patients (Fig. 5A).[73] The subpopulation of tumour-initiating cells identified in human breast cancer exhibit CD44$^+$ and CD24$^-$ cell-surface markers, and were found to be more resistant to conventional therapies than the more differentiated cancer cells,[74] suggesting that tumour relapse can be accomplished by these tumour-initiating cells after treatment (Fig. 5B). Therefore, CSC's must be eliminated to affect a cure on cancer (Fig. 5C).

As cancer stem cells are proposed to be more resistant to conventional therapies, it becomes necessary to find new ways to target them in order to treat cancer and prevent its recurrence. The central role that EMT has in tumour progression makes it an obvious target for therapeutic intervention. However, the inhibition of EMT may also have serious consequences for wound healing processes and also tissue remodelling during repair and regeneration.

Independent groups have demonstrated that cells that have undergone EMT exhibit stem-cell characteristics, including resistance to chemotherapy.[68,70,71,75] Therefore targeting EMT inducers may help treating cancer. Olmeda *et al.*[76,77] have shown that by silencing Snail with shRNA, they can block EMT by enabling E-cadherin expression and lead to a MET process. Knowing that EMT plays a main role in generating cancer stem cells is crucial in order to target its different pathways, and screening with different drugs to block this feature. The development of new therapies blocking EMT and its precursors (transcription factors) is of vital importance in this quest for curing cancer and avoid its progression. In different approaches regarding the blockage of EMT during cancer progression (tumour growth and proliferation), it has been shown that blocking oncomirs (microRNAs) can be a useful resource as described by Yan *et al*[78] after inhibiting proliferation of human breast cancer cells (MCF7) *in vivo* by miR-21 knockdown with peptide nucleic acids (PNAs).

If this crosslink between EMT and cancer stem cells is confirmed, treatment of cancer at early stages should include EMT blocking agents that could inhibit TGF-β downstream effectors like ZEB1.

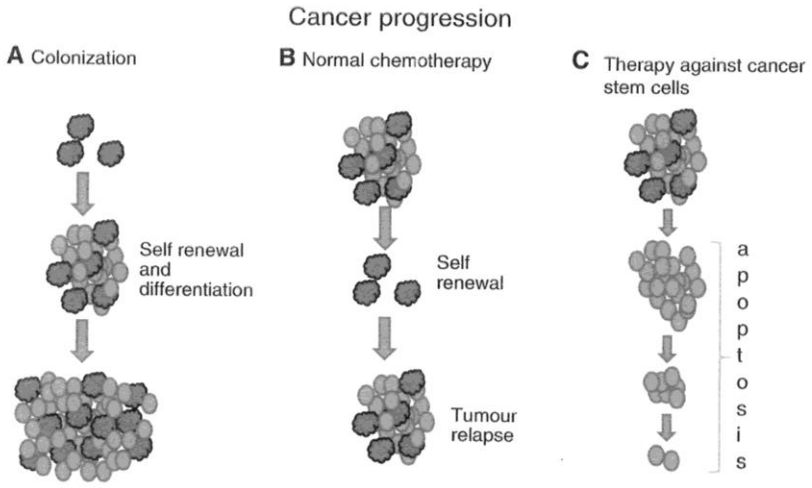

Figure 5. Cancer stem cells are resistant to conventional therapy. (A) Self-renewal and differentiation from single cancer stem cells (irregularly shaped) to differentiated cancer cells or progeny (round shaped). (B) Tumour relapse after conventional chemotherapy against differentiated cancer cells. (C) If cancer stem cells are eliminated, then their progeny would pass through a period of multiple apoptosis, and the result would be a slowly decrease in tumour cells until the tumour disappears completely.

By these means, resistance to apoptosis and self-renewal characteristics can be nullified, preventing not only migration of cancer cells, but also the development of the primary tumour by stopping cancer stem-cells multiplication (Fig. 6).

Cellular reprogramming, EMT and E-cadherin

Cell reprogramming can be defined as the ability of specific transcription factors to change the identity of specialized differentiated cells into different cell lineages.[79,80] As described by Yamanaka *et al.*, it might also be an important step in the acquisition and maintenance of pluripotency of epithelial cells achieved by defined pluripotency transcription factors: OCT4, SOX2, KLF4 and c-MYC (OSKM), also known as the Yamanaka cocktail.[81,82] This group showed that pluripotency can be induced in mouse embryonic fibroblasts (MEFs), and these can be reprogrammed by the above mentioned factors to become induced pluripotent stem cells (iPSCs), and this mechanism would be named cell reprogramming to pluripotency.[82]

Stadtfeld *et al.*[83] described that an MET is necessary for the reprogramming process, which was then confirmed by Redmer *et al.*,[84] showing that E-cadherin might play an important role in maintaining pluripotency in mouse embryonic stem cells (mESCs), and it can also replace OCT4 during reprogramming after an MET. It is not well understood how E-cadherin maintains pluripotency. However, its role was confirmed by replacing OCT4 in the OSKM combination with an E-cadherin-expressing retrovirus (pMXs-Ecad) which demonstrated that this new combination of factors (ESKM) reprogrammed MEFs into iPSCs, the latter being able to form teratomas in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, while SKM clones just formed small lumps.[84]

The metastatic progression of different types of cancer is carried out by a complex network of pathways in which E-cadherin plays different roles regarding EMT, MET and as mentioned above, cell reprogramming (Fig. 7). The role of E-cadherin in cell reprogramming as a pluripotency inducer could be one of the essential features of metastasis due to its ability to change cellular identity once the cancer cells have reached their new niche after EMT and subsequent MET (in which E-cadherin is highly upregulated).

Furthermore, Liao *et al.*[85] showed that cell reprogramming through an MET could also be carried out by microRNA (miRNA) expression in mesenchymal cells. They demonstrated that

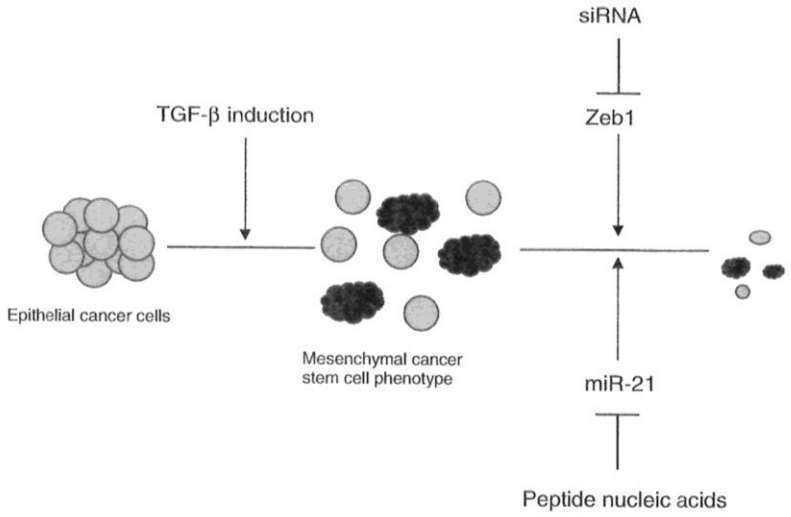

Figure 6. Therapeutic intervention. This diagram shows two different possible therapeutic levels regarding the blockage of EMT precursors and miR-21 as an oncomir. After inhibiting ZEB1 and/or miR-21 cancer stem cells would lose their acquired stem-cell characteristics and would start dying by apoptosis and stop dividing.

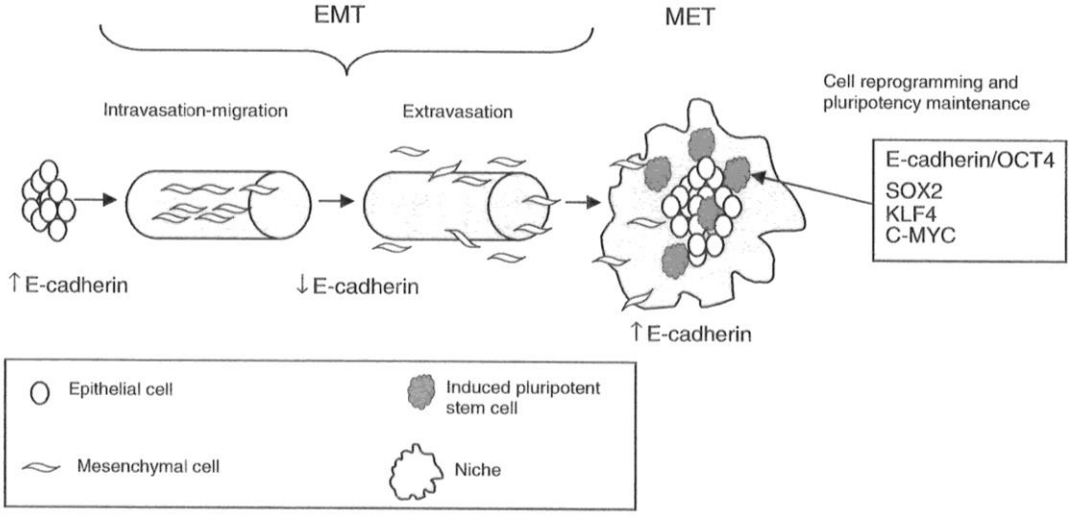

Figure 7. Cell reprogramming. After epithelial cells acquire mesenchymal characteristics by downregulation of E-cadherin during EMT and migrate through blood and lymphatic vessels, they colonize their new target organ (niche) while E-cadherin is upregulated and enable cells to return to their epithelial phenotype through a MET. While these cells colonize their new niche, E-cadherin and defined pluripotency factors (OCT4, SOX2, KLF4 and c-MYC) reprogram cells to become pluripotent stem cells.

overexpression of miR-302 complex in addition to miR-367 in fibroblasts was enough to downregulate a TGF-β receptor (TGF-βR2), thus, enabling cells to undergo a MET by upregulation of E-cadherin and cell reprogramming into iPSCs.

These findings would confirm a link between the epithelial phenotype and pluripotency, elucidating potential targets for prevention or treatment of metastatic consequences.

EMT in companion animals

Epithelial–mesenchymal transition in companion animals (dogs and cats) has not been widely studied. Chandler *et al.*[86] showed the importance of Slug as a transcription factor during cell migration in wound healing. They used 12 dogs to which they induced corneal wounds and measured Slug expression in wounded and unwounded corneas.

They assessed the rate of wound healing with and without Slug transfection. Their results showed an upregulation of Slug in wounded corneas compared to the unwounded ones. They also observed downregulation of E-cadherin and β-catenin (epithelial markers) in healing tissues, meaning that transcription factor Slug downregulated these epithelial markers so the cells could lose their adherens junctions and start moving through the cornea. Finally, when transfecting wounded corneas *ex vivo* with Slug, the migration rate increased dramatically, suggesting that epithelial cells became mesenchymal through an EMT process.

In two different studies, Aresu *et al.*[87,88] have been studying the role of EMT in canine renal fibrosis, in which EMT has been found to be essential to generate this abnormality that can cause several renal diseases. They showed that during renal inflammation-fibrosis, leading to diseases such as glomerulonephritis, epithelial markers E-cadherin and β-catenin[87] and cytokeratin[88] are downregulated whilst the mesenchymal marker vimentin is identified and upregulated at inflammation areas. As we can see, these research groups have found important results regarding type II EMT (wound healing and fibrosis), but these studies have yet to be extended to type III EMT (cancer progression and metastasis).

Several authors from different research groups have published interesting results regarding expression of cell adhesion molecules and their correlations with tumour growth and cell proliferation.

Han *et al.*[89] observed an increased expression of β-catenin accumulation in the cytoplasm and nucleus in canine cutaneous melanotic tumours. Normally, β-catenin is degraded in the cytoplasm; however, dysregulation of the Wnt/β-catenin signalling pathway leads to accumulation of this protein, causing uncontrolled cell proliferation.[90] These findings have been also documented in canine colorectal tumours[91] and canine osteosarcoma.[92] Moreover, Nowak *et al.*[93,94] compared the reciprocal relations between extracellular matrix metalloproteinase (MMP-9), E-cadherin, the proliferation associated antigen Ki-67 and β-catenin in canine mammary adenocarcinoma, finding that the decreased expression of E-cadherin is inversely proportional to the expression of MMP-9, Ki-67 and nuclear-located β-catenin, whereas a direct correlation was observed between MMP-9 and Ki-67, and β-catenin and Ki-67. They also observed increased cell growth and proliferation associated with higher expression of nuclear accumulation of β-catenin.

In canine colorectal adenocarcinoma, Aresu *et al.*[95] observed lower expressions of E-cadherin and β-catenin in the cell membrane in the majority of analyzed tumours. They found a correlation between low expression of E-cadherin with higher grade (grade 4) tumours and higher mean age of patients. Reduced expression of cell membrane accumulation of β-catenin was also related to tumours with higher grade, but also with increased tumour size.

Furthermore, Ide *et al.*[96] found that increased expression of cell adhesion molecules N-cadherin, doublecortin and nuclear β-catenin was closely associated with progression of canine meningioma.

Most of these groups correlate the expression of these molecules associated with cancer cell growth and/or progression, but interestingly, they do not make any observation about EMT type III in small animals, nevertheless, all of their perspectives are of great importance to the development of deeper understanding of EMT in cancer development and progression.

In the authors' laboratory, we have studied EMT as a feature of human, canine and feline mammary carcinoma and feline squamous cell carcinoma in an attempt to elucidate the role of EMT in cancer progression and in the acquisition of stem-cell-like characteristics. We have demonstrated that dog and cat cells undergoing EMT by TGF-β stimulation show mesenchymal morphology (spindle shapes) and lose cell–cell contact, separating from the group of cells. These cells also overexpress mesenchymal markers (fibronectin and vimentin) while epithelial markers (E-cadherin and β-catenin) are downregulated. Interestingly, cells that underwent EMT (verified by protein expression and morphologic changes) are more capable of forming spheres, which are representative of cancer stem cells.[97]

These results offer the opportunity for exploring EMT as a potential therapeutic target in companion animal oncology.

Unanswered questions and future work

There is still much to be done regarding this difficult task of knowing and understanding the complex network of cancer and its progression mechanisms.

Some of the key questions include:

- What other mechanisms and factors are involved in EMT and MET?
- Can this process be targeted therapeutically and safely?
- What mechanisms link EMT, MET and pluripotency in cells?
- What is the role of microRNA's in these processes and can they be targeted

It is becoming clear that EMT and MET are key mechanisms in cancer progression. However, it is also clear that these processes may also be involved in the maintenance of pluripotency in normal cells. One would anticipate that there will be convergence of these respective research fields and it is likely that key questions will be answered by studying both normal and neoplastic processes simultaneously.

Conclusion

EMT and cancer progression is an emerging field of cancer research. EMT may be very relevant to cancer induction and progression research. Moreover, if we can confirm that EMT/MET process is an inducer of stem-cell characteristics, then we would be able to generate new therapies against cancer and its progression and metastasis.

Acknowledgements

This work was supported by the Mexican National Council on Science and Technology (CONACYT) and the Scottish Overseas Research Students Award Scheme (SORSAS).

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 Veterinary and Comparative Oncology, doi: 10.1111/j.1476-5829.2011.00313.x

Appendix 2: Canine mammary carcinoma cells microRNA full screening. Data included in CD.

Folder 1: Normalised counts of the full miRNA screening

Folder 2: Counts for miRNA expressed after day1 of TGF-β stimulation

Folder 3: Counts for miRNA expressed after day 10 of TGF-β stimulation

Folder 4: Counts for miRNA expression after day 17 of TGF-β stimulation

Folder 5: Counts for miRNA expression after day 23 of TGF-β stimulation

Folder 6: Counts for miRNA expression after TGF-β withdrawal at day 23

Folder 7: counts for miRNA expression after TGF-β withdrawal, compared with TGF-β-stimulated samples.